The enigma of dual reproduction in potato

Casting light on tuberization and flowering

Faline Dawn-Marie Plantenga

Thesis committee

Promotor
Prof. Dr Leo F.M. Marcelis
Professor of Horticulture and Product Physiology
Wageningen University & Research

Co-promotors
Dr Ep Heuvelink
Associate professor, Horticulture and Product Physiology
Wageningen University & Research

Dr Christian W.B. Bachem
Assistant professor, Plant Breeding
Wageningen University & Research

Other members
Prof. Dr Salomé Prat, Centro Nacional de Biotecnología – CSIC, Madrid, Spain
Prof. Dr Paul C. Struik, Wageningen University & Research
Prof. Dr Richard G.H. Immink, Wageningen University & Research
Dr Pim Lindhout, Solynta, Wageningen

This research was conducted under the auspices of the C.T. de Wit Graduate School for Production Ecology and Resource Conservation

The enigma of dual reproduction in potato

Casting light on tuberization and flowering

Faline Dawn-Marie Plantenga

Thesis

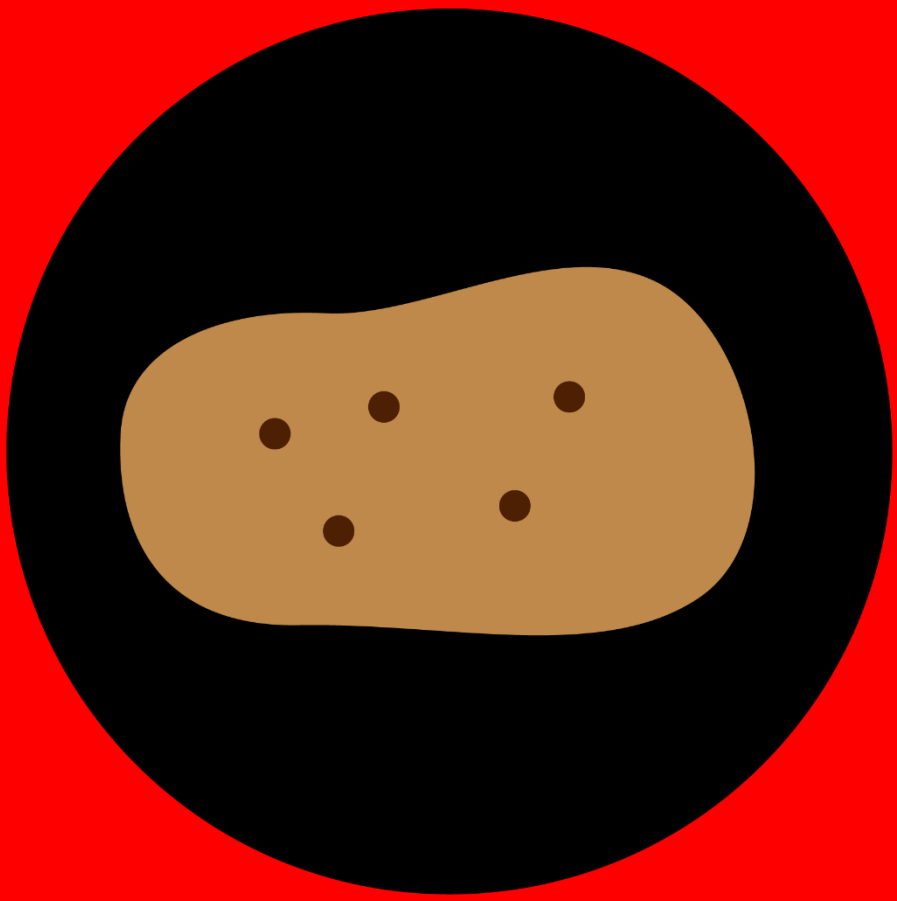
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 12 February 2019
at 4 p.m. in the Aula.

Faline Dawn-Marie Plantenga
The enigma of dual reproduction in potato. Casting light on tuberization and flowering, 187 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2019)
With references, with summaries in English and Dutch

ISBN 978-94-6343-394-5
DOI https://doi.org/10.18174/466260

Table of contents



1. General introduction

The potato plant

Potato as a food crop

With a rapidly growing human population, intensifying food production to meet the growing consumption is a major point of focus in agriculture. Potato is one of the largest agricultural crops and in terms of human consumption, potatoes are only surpassed by rice and wheat (International Potato Center, 2018). Worldwide, potato production has increased with 14% over the past decade (2006-2016, http://faostat.fao.org), which in part is due to a strong increase of potato production in developing countries (22% 2006-2016, http://faostat.fao.org). Potato plants are grown in a wider range of latitude, altitude and climatic zones than any other major food crop due to their high agronomical plasticity and yield potential (Horton and Anderson, 1992; Celis-Gamboa, 2002; Hirsch *et al.*, 2013). The high nutritional value of potato compared to other staple foods may explain its rise in popularity. Potato tubers contain relatively high levels of carbohydrates and proteins, and are a source of vitamins and minerals (Rodríguez-Falcón *et al.*, 2006; Zaheer and Akhtar, 2016). Potato cultivation started in the Andean highlands under cool temperatures and relatively short day lengths (Prat, 2010). The potato varieties that originated in the Andes (*Solanum tuberosum* L. ssp. *andigena*) tuberize under short-day conditions. Modern cultivated potato derives from Chilean landraces (*Solanum tuberosum* L. ssp. *tuberosum*), that grow in the lowlands of southern Chile and are adapted to longer day lengths (Sukhotu *et al.*, 2005; Rodríguez-Falcón *et al.*, 2006; Prat, 2010). To optimize potato production, a greater understanding of potato plant growth is required.

Potato reproduction

Potato plants can reproduce sexually by forming flowers, berries and seeds. At the same time potato plants can reproduce asexually by forming underground stems called stolons, which swell and become tubers. How two reproduction processes are regulated simultaneously remains to be understood. Potato represents a good model crop to study this dual reproduction.

Tuberization is the reproduction method that has been most studied in the potato plant. Tubers are the only edible part of the potato plant (Friedman *et*

al., 1997), and tubers are used for plant propagation. Tubers that are used to grow new potato plants are called seed tubers. Vegetative propagation with seed tubers is not without risk; viruses and other pathogens like *Phytophthora infestans* are spread through seed tubers (Jansky *et al.*, 2016). Special efforts that keep seed-tuber material free of disease are expensive, arduous and time consuming and even with the best efforts, diseases often persist (Ewing and Struik, 1992). An alternative to vegetative propagation is the use of seeds, in potato referred to as true potato seeds (TPS). In contrast to vegetative reproduction, using TPS as starting material limits disease transferal to the next generation (Ewing and Struik, 1992; Jansky *et al.*, 2016). Additionally, TPS are a lot smaller than seed tubers, making them easier and cheaper to transport and store. However, TPS are not yet used as propagation material, mainly because potato is a very heterozygous crop. Therefore crossing two parent plants will lead to a progeny with varying genetic backgrounds, making it impossible to predict which traits the next generation will have.

Fortunately hybrid breeding is starting to be implemented in potato. Hybrid breeding makes use of crosses between homozygous diploid parent lines (Lindhout *et al.*, 2011). Because both parent lines are homozygous, TPS of the progeny will have the same genetic material each time a cross is made and can therefore be used for propagation. Hybrid breeding in potato is not only advantageous for TPS, but allows for the deliberate crossing of desired traits into a new variety. Deliberately crossing traits into the next generation is in contrast to conventional potato breeding with heterozygous parent lines. In conventional potato breeding breeders have to make many crosses and raise around 100.000 seedlings to select for one new variety with desired traits (Lindhout *et al.*, 2011). Hybrid breeding has not been implemented in potato before because next to being heterozygous, commercial genotypes are tetraploid. Furthermore, self-crossing (selfing), which is needed to make homozygous lines, leads to inbreeding depression (Hirsch *et al.*, 2013). These traits make the development of homozygous parent material extremely difficult in tetraploid potatoes. Using diploid potatoes would be much more convenient, but diploid potatoes are self-incompatible (Jansky *et al.*, 2016). Fortunately, the introduction of a *Sli* gene from a wild diploid potato eliminates self-incompatibility in diploid potatoes (Hosaka and Hanneman, 1998), enabling selfing and the generation of homozygous parent material. Consequently, hybrid breeding can now be implemented in potato using diploid potato plants.

Hybrid breeding in potato and use of TPS have increased the interest in potato flowering. In contrast to tuberization, very little scientific research has been done on potato flowering (Almekinders and Struik, 1996). In this thesis we aim to increase the knowledge on potato flowering, and tuberization. A coordinated regulation of flowering and tuberization time is essential for successful survival of the potato plant. However, it is not known how these two processes are co-regulated. How does a potato plant decide whether to tuberize, flower or both? Can these reproduction processes be controlled separately? Do these two modes of reproduction compete with each other? Studying flowering and tuberization simultaneously will give us new information on this plant developmental control.

Tuberization versus flowering

Separating tuberization and flowering

In certain circumstances tuberization may be preferred while in others fast and abundant flowering may be required. For high potato yields, it is advantageous for tuberization to only begin after the shoot has grown enough to provide sufficient assimilates during tuber growth. Conversely, when the growing season is short, it may be desired to induce early tuberization to allow tuber bulking before the end of the growing season. In the case of potato breeders, abundant flowering is desired. However, tubers are strong assimilate sinks which may inhibit flowering (Sweetlove *et al.*, 1998). Therefore, optimal flowering may be achieved by accelerating flowering time or inhibiting tuberization. Also, potato breeders wish for fast flowering to decrease the time needed to complete several reproduction cycles, which can accelerate the breeding process. Being able to separately control tuberization and flowering time can optimize potato production and breeding and ultimately increase and improve the world food supply.

A flexible environmental switch to induce either tuberization or flowering

How can potato tuberization and flowering be controlled for optimal production and breeding? It is possible to select for new varieties which have the desired

tuberization or flowering properties. For potato production this may be a variety that tuberizes early and has poor flowering. In contrast to species where the yield of the fruit is important and is dependent on the flowering success, potato flowers are of no interest to the grower. Breeders, however, prefer varieties with abundant and fast flowering to allow fast and successful crossing, but these varieties may not have the best tuberization, which is needed for potato production. Ideally, tuberization and flowering should be controlled using a flexible switch. The environment could be used for this purpose. By changing the conditions in which potato plants grow, plant development is altered. Controlling growing conditions for optimal tuber yield may be difficult, as most potato production is done in the open field. However, breeding often takes place in controlled environments, in which optimal growing conditions for potato flowering could be implemented. Before the environment can be used to control the reproduction in potato, it must be understood how the environment affects tuberization and flowering.

How are tuberization and flowering regulated by the environment?

Environmental cues

Whether plants transition from vegetative growth to reproductive development, depends on various endogenous and exogenous signals (Bernier *et al.*, 1993; Srikanth and Schmid, 2011). Plants use seasonal cues to determine when conditions are optimal to ensure reproductive success. Potato reproduction has been found to be regulated by various environmental cues like light, temperature, nutrient availability, water content and CO_2 concentration (Werner, 1935; Gregory, 1956; Ewing and Struik, 1992; Temmerman, 2007; Schafleitner *et al.*, 2011).

It is known that high temperatures and high nitrogen levels can inhibit tuberization (Ewing and Struik 1992; Jackson 1999) and high temperatures accelerate flowering time and can improve flower bud development (Jones and Borthwick, 1938; Ewing and Struik, 1992; Almekinders and Struik, 1994, Almekinders and Struik 1996). Furthermore, some work has been done on other environmental cues like CO_2 concentration and water supply (Temmerman,

2007; Schafleitner *et al.*, 2011). However, the strongest and best described environmental factor influencing potato development is light.

Light

Light can be separated into several environmental regulators: day length (also called photoperiod), spectrum, intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$) and the daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$).

Photoperiod

Many plants use the photoperiod as an environmental cue to regulate flowering (Imaizumi and Kay, 2006). In potato, short days, or more accurately long nights induce tuberization (Gregory, 1956; Ewing and Struik, 1992; Jackson, 1999; Rodríguez-Falcón *et al.*, 2006). Although short days usually have a lower DLI than long days, the photoperiod is the cause for tuber induction. Plants grown in short days supplemented with very low levels of light to extend the photoperiod, receive similar DLIs as plants grown in short days, but are unable to tuberize (Chapman, 1958). The photoperiod is perceived by the leaves, and under short-day conditions a tuberization signal is transported to the underground stolons (Rodríguez-Falcón *et al.*, 2006). Once induced, stolon tips stop elongating and start to swell and form tubers (Jackson 1999). If the plant is placed in non-inductive conditions, tuber swelling stops, stolon growth continues and eventually stolons grow out of the soil to form new shoots (Chapman 1958; Jackson 1999; Rodríguez-Falcón *et al*., 2006). In contrast to *S. tuberosum* ssp. *andigena* (*S. andigena*) plants that require day lengths shorter than 12 hours to tuberize (Ewing and Struik, 1992), cultivated potatoes belonging to the *S. tuberosum* ssp. *tuberosum* (*S. tuberosum*) are able to tuberize under longer day lengths, but still have accelerated tuberization under short days (Ewing and Struik, 1992; Rodríguez-Falcón *et al.*, 2006; Abelenda *et al.*, 2014). Accelerated tuberization does not necessarily mean a higher tuber yield, as later tuberization allows the plant to first invest in leaf area and acquire more assimilates which later increase tuber yields (Werner, 1935).

In contrast to tuberization, the photoperiodic effect on potato flowering is less clear. The problem is not only the lack of information, but also the inconsistencies between reports (Almekinders and Struik, 1994). The most important inconsistency is due to the variation in the definition of “flowering”.

While some authors investigated flowering time as the time until initiation of the floral buds (e.g. Firman *et al.,* 1991; Almekinders and Struik, 1994; González-Schain and Suárez-López, 2008), most authors considered flowering time as the time until anthesis (open flowering stage). However, when only scoring anthesis, not only initiation of the flower primordia is considered, but also development of the flower primordia, which are two distinct processes (Almekinders and Struik, 1996). Additionally, most studies on potato flowering vary photoperiod by extending the day length. Consequently, photoperiod effects are mixed with DLI effects, leading to unreliable results when considering photoperiodic regulation.

Despite the variation found between photoperiodic effects on potato flowering, some common observations have been made. Photoperiodic regulation of flowering is not as strong as photoperiodic regulation of tuberization (Jackson and Thomas, 1997). In fact, Jones and Borthwick (1938) discovered that potato plants initiate flower primordia in long and short day lengths and even under complete darkness. Although photoperiod is not a requirement for flower initiation, it could still affect the speed at which this process takes place. Most studies report that flowering time, as measured by the number of leaves formed before the inflorescence, is day neutral (Firman *et al.*, 1991; Almekinders and Struik, 1994; Almekinders and Struik 1996; González-Schain *et al.*, 2012). However, both long-day flowering (less leaves in long days) and short-day flowering (less leaves in short days) have been reported as well (Jones and Borthwick, 1938; Almekinders and Struik, 1994). In the case of long-day flowering, longer days also received a higher DLI than short days. Flowering time measured in days ranged from day neutral (González-Schain and Suárez-López, 2008) to long day (González-Schain *et al.*, 2012), but again longer days had a higher DLI. Although the effect of photoperiod on flowering time is unclear, photoperiod does have a clear effect on flower development. Long days improve the number of inflorescences, the number of leaf primordia per inflorescence and the number of open flowers (Driver and Hawkes, 1943; Zafar, 1955; Pallais, 1987; Almekinders, 1992; Ewing and Struik, 1992; Almekinders and Struik, 1994; Macháčková *et al.*, 1998; Markarov, 2002; Schittenhelm *et al.*, 2004).

To summarize, tuberization is a short-day process. There are inconsistencies concerning the effect of photoperiod on flowering time, though most reports propose flowering time to be day neutral in potato. Flower development is improved in long days.

Light spectrum

In studies testing the photoperiod, red and far-red light have been used to test the photoperiodic effect on tuberization. White or red light applied in the middle of the long night, eliminated the short-day tuberization induction, but application of far-red light immediately after this "night break" recovered the short-day tuberization response (Batutis and Ewing, 1982). It is not yet known how light spectrum applied during the day affects tuberization, with the exception of a research describing the effect of continuous blue light applied to potato plants. This treatment was able to inhibit tuberization (Fixen *et al.*, 2012). An effect of light spectrum on potato flowering is unknown.

Daily light integral and light intensity

The effect of light intensity on tuberization was demonstrated growing plants under different light intensities. A reduced intensity delayed tuberization time and the number of tubers formed (Demagante and Zaag, 1988). However, reducing the light intensity without altering the day length means the DLI was also reduced. Thus the negative effect of light intensity on tuberization could also be caused by a low DLI.

As mentioned previously, longer days with a higher DLI often had a positive effect on flowering time. A direct effect of light intensity or DLI on potato flowering has not been studied. However, an indirect effect of light intensity and DLI was described by Firman *et al.,* (1991). The authors found that climate chamber conditions, where light intensity was much lower than in the field, increased the number of leaves formed before the inflorescence and in one genotype inhibited flower initiation altogether. This last result is in contrast to earlier findings which demonstrate that flower initiation can take place in total darkness (Jones and Borthwick, 1938). Furthermore, when comparing between climate chamber and field, not only light intensity and the DLI differ. Many other factors i.e. light spectrum could have caused and effect, thus it is not certain what the effect of light intensity and the DLI on flowering time is. The effect on flower development is more evident; increasing light intensity and the DLI decreased flower bud abortion, and allowed more flower primordia to develop into open flowers (Driver and Hawkes, 1943; Demagante and Zaag, 1988; Ewing and Struik, 1992).

Summarizing the light effects on tuberization and flowering

Light has a distinct effect on tuberization; short days and high light intensities or DLIs promote tuberization. Furthermore far-red light can relieve red light inhibition of tuberization, while blue light can inhibit tuberization. Flowering time may be day neutral, and higher light intensity and DLI may accelerate the time till flowering. However, flowering time must be studied in more detail to confirm these indications. Flower development is accelerated by high light intensity or high DLI. The effect of light spectrum on flowering must still be elucidated. Although information is available on regulation of potato reproduction by light, much still needs to be elucidated, especially concerning flowering.

Interaction between tuberization and flowering

The environmental cues that stimulate flower development, such as higher temperatures and longer days, generally lead to an increase in shoot assimilate supply due to delayed tuberization (Almekinders and Struik, 1996). Although potato plants simultaneously form leaves, tubers, flowers and berries, tuberization and shoot growth are generally considered to be competing processes (Almekinders and Struik, 1996). Once tuberization starts, assimilate partitioning shifts from the shoot to the tubers, and due to a shortage of assimilates in the shoot, recently formed flower buds cannot develop, and abort (Almekinders and Struik, 1996). Therefore, earlier tuberization would mean a bigger detrimental effect on flowering (Pallais, 1987). Because flower initiation takes place before tuberization (Firman *et al.*, 1991), it is expected that only flower development is affected by tuberization. Breeders have regularly tried to improve potato flower development by removing the tubers, with varying success (Thijn, 1954; Jessup, 1958; Abdel-Wahab and Miller, 1963; Weinheimer and Woodbury, 1966). As there are contrasting results concerning competition between flowering and tuberization, it must be discovered if competition is really taking place. Once this has been investigated, it can be determined if there is a direct environmental effect on flowering or if this is regulated through tuberization.

Light as an environmental switch

The various effects of light on potato reproduction make this environmental cue suitable as an environmental switch to control tuberization and flowering when necessary. Light is a convenient switch to use in controlled environments like greenhouses and climate chambers. With the arrival of light emitting diodes (LEDs) it is becoming increasingly easy to manipulate light conditions to create optimal conditions for plant growth and development (Massa *et al.*, 2008; Ouzounis *et al.*, 2015). Before studying the effect of light on tuberization and flowering in more detail, we must increase our knowledge on the mechanisms regulating of tuberization and flowering. With this knowledge we can optimize our light recipes of varying photoperiods, light spectra, light intensities and DLIs to make a light environment that is optimal for tuberization or flowering.

The molecular regulation of tuberization and flowering by light

Photoperiod

The molecular regulation behind photoperiodic tuberization is starting to be unraveled. Potato tuberization shares many similarities with the flowering control in other species. This is not unexpected, as large parts of the photoperiodic flowering pathway are conserved between species (Matsoukas *et al.*, 2012). The extensively studied *Arabidopsis* can act as a model for the molecular control behind tuberization in potato (Izawa *et al.*, 2003; Andrés and Coupland, 2012). However, as Arabidopsis is a long-day and potato a short-day plant, and two different developmental processes are affected, distinct differences in regulation may be expected (Song *et al.*, 2010). A key player in photoperiod regulated flowering in *Arabidopsis* is *CONSTANS* (*CO*). How the regulation of *CO* determines photoperiodic flowering is reviewed in Andrés and Coupland (2012) and Song *et al.* (2014). In long days CO activates *FLOWERING LOCUS T (FT)*, a mobile signal which travels from the leaves to the apical meristem to induce flowering. Flowering is only induced in long days due to several levels of control on *CO.* The *CO* gene is under the control of a circadian

clock component called *GIGANTEA (GI)*. Under long day conditions GI interacts with FLAVIN KELCH F BOX 1 (FKF1) and together this complex degrades an inhibitor of *CO* expression called CYCLING DOF FACTOR (CDF). Thus, in long-day conditions, CO is expressed at the end of the day, while in short days GI does not form a complex with CDF and *CO* expression is repressed in the day and only peaks in the night. An additional level of control takes place on the protein level. In short days, *CO* is expressed in the night and in the absence of light the CO protein is degraded by a CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYTOCHROME A (SPA1) complex. In long days, due to the action of GI and FKF1, *CO* is expressed in the day and under these light conditions, the COP1-SPA1 complex is degraded by active photoreceptors CRYPTOCHROME 2 (CRY2) and PHYTOCHROME A (PHYA). Therefore, due to the coincidence of light and *CO* expression, the CO protein can only be present in long days to activate *FLOWERING LOCUS T (FT)* transcription and induce flowering (Andrés and Coupland, 2012).

A homolog of *CONSTANS* in potato *(StCOL1)* is central for the photoperiodic control of tuberization (González-Schain *et al.*, 2012; Abelenda *et al.,* 2016). As opposed to Arabidopsis CO, potato StCOL1 does not induce, but inhibits tuberization (Martínez-García *et al.*, 2002; Navarro *et al.*, 2011; González-Schain *et al.*, 2012). *StCOL1* is under circadian control and is expressed in both short and long days in the leaves (González-Schain *et al.*, 2012). *StCOL1* expression is regulated by the same group of conserved genes which also function in the photoperiodic flowering control of *Arabidopsis* (Kloosterman *et al.*, 2013). Potato homologs *StGI*, *StFKF1,* and *StCDF1* play a central role in control of tuberization as their transcripts act on *StCOL1* (Kloosterman *et al.*, 2013; Abelenda *et al.*, 2014). It was found that allelic forms of *StCDF1* that were truncated in the StFKF1 interaction domain, led to a constitutive repression of *StCOL1* because StFKF1 was unable to bind to StCDF1 to trigger degradation of StCDF1 (Kloosterman *et al.*, 2013). This truncation of the StCDF1 is likely to form the basis of domesticated potatoes that are not obligate short-day plants and are able to tuberize in longer photoperiods as well (Kloosterman *et al.*, 2013).

In the obligate short-day *S. andigena*, the peak expression of *StCOL1* in long-day conditions coincides with the light period, which is suspected to be key for the inhibiting effect of StCOL1 on tuberization in long days (Abelenda *et al.*, 2016). In the light period, the photoreceptor phytochrome B (PHYB) is active and functions as a stabilizer of the StCOL1 protein, in contrast to Arabidopsis CO

which is stabilized by CRY and PHYA (Andrés and Coupland, 2012; Abelenda *et al.*, 2016). However, *StCOL1* is also expressed in the light period of a short day, in which tuberization is not inhibited. It has been suggested that peak expression of *StCOL1* must coincide with light to inhibit tuberization (Song *et al.*, 2014; Navarro *et al.*, 2015; Abelenda *et al.*, 2016). In a short day, peak *StCOL1* expression takes place in the night, thus active PHYB in the light may only stabilize low levels of StCOL1, which still permit tuberization.

In long days StCOL1 induces the expression of an *FT* homolog called *StSP5G,* which in potato acts as a repressor of tuberization (Potato Genome Sequencing Consortium 2011; Kloosterman *et al.*, 2013; Abelenda *et al.*, 2016). StSP5G inhibits expression of another homolog of *FT* called *StSP6A,* which encodes for the tuberization signal (Potato Genome Sequencing Consortium, 2011; Navarro *et al.*, 2011; Abelenda *et al.*, 2016). After *StSP6A* is expressed, StSP6A travels as a mobile signal from the leaves to the stolons of the plant, where tuberization is initiated (Navarro *et al*., 2011). Once StSP6A arrives in the stolons, the StSP6A signal is thought to be enhanced by an auto-regulatory loop, where StSP6A triggers local expression of *StSP6A* (Navarro *et al.*, 2011). Next to the tuberization signal *StSP6A,* an additional tuber inducer called StBEL5 is thought to control tuberization in response to photoperiod. BEL1-like transcription factors regulate several growth and development processes, and in potato, StBEL5 regulates tuber formation (Chen *et al.*, 2003). StBEL5 RNA may act as a mobile signal by moving from the leaves to the stolons to induce tuberization (Banerjee *et al.*, 2006). *StBEL5* expression is induced by low levels of blue and red light, but not by the photoperiod. However, the stabilization and transport of StBEL5 RNA is mediated by two poly-pyrimidine tract-binding (PTB) proteins, that are most active in short days (Cho *et al.*, 2015; Hannapel *et al.*, 2017). It was proposed that StBEL5 acts on *StSP6A* expression in both the leaves and in the stolons, and thus may influence the auto-regulatory loop of *StSP6A* (Hannapel *et al.*, 2017). Thus, due to the short-day regulated stabilization and localization of StBEL5, photoperiod has another level of control on tuberization.

In contrast to photoperiodic tuberization control, almost nothing is known about the molecular mechanisms behind potato flowering. A third *FT* homolog called *StSP3D* has been discovered and silencing of this gene leads to delayed flowering (Potato Genome Sequencing Consortium, 2011; Navarro *et al.*, 2011). Even though flowering has been described as day-length neutral, *StSP3D*

may be under photoperiodic control. *StSP3D* expression is higher in short days compared to long days, as is the case for *StSP6A* (Navarro *et al.*, 2011). This leads to the question how potato flowering is induced in long days. It has been proposed that in contrast to tuberization in the stolons, where an auto-regulatory *StSP6A* expression loop enables large quantities of StSP6A to be produced, the shoot apical meristems are very sensitive to StSP3D and even very low expression in long days is enough to initiate flower initiation (Abelenda *et al.*, 2014). Thus, despite the photoperiodic regulation of *StSP3D*, flowering could still be day-length neutral as low StSP3D levels in long days may also initiate flowering. However, this is only a theory and has to be studied in more detail. Furthermore, it is not yet clear if all the genes known to act on *StSP6A* also act on *StSP3D,* or whether the flowering pathway diverges from the tuberization pathway at some point. It was found that lines overexpressing or silenced in *StCOL1* both had an accelerated flowering time (González-Schain *et al.*, 2012), which indicates that StCOL1 does play a role in *StSP3D* induction, yet how remains to be discovered.

In summary, a lot is known about photoperiodic tuberization. It is known which genes are responsible for tuberization control and it is known how they are expressed throughout long and short days. However, it is not yet fully understood why long days inhibit tuberization in the obligate short-day *S. andigena*, but short days induce tuberization. Although a coincidence model alike that in Arabidopsis has been proposed for tuberization, *StCOL1* is expressed in both long and short days in potato, which is not the case for Arabidopsis. As suggested previously, coincidence between light and peak *StCOL1* expression may be key in inhibiting tuberization. Finding out if a coincidence model really applies in potato, will be investigated in this thesis.

For flowering, a lot still needs to be discovered. How can *StSP3D* be regulated by short days but flowering be day neutral? Is flowering really day length neutral? In short, study on the photoperiodic effect on flowering is still necessary to increase our understanding of these processes in potato, which will be done in this thesis.

Light spectrum

Although little research has been done on light spectrum, indirectly information on regulation by light spectrum is available by studying the photoperiodic

tuberization pathway. Differences in light spectrum are sensed by photoreceptors in the plant. As mentioned in the previous section, photoreceptor PHYB plays an important role in tuberization control. PHYB is one of several phytochromes present in potato (Jackson *et al.*, 1996; Yanovsky *et al.*, 2000). Phytochromes exist in two photo-convertible forms, which react to differences in light spectrum. The active Pfr form is converted into the non-active Pr form by far-red light, while the non-active Pr form is converted back to the active Pfr form by red light (Casal, 2000). In the case of tuberization, active PHYB (Pfr form) stabilizes StCOL1, thereby inhibiting tuberization (Abelenda *et al.*, 2016). By silencing *PHYB*, potato plants are able to tuberize in non-inducing long-day conditions (Jackson *et al*., 1996).

Next to red and far-red light, blue light was shown to affect potato tuberization. Fixen *et al.* (2012) demonstrated that continuous blue light inhibited tuberization, but this was genotype specific. The authors suggested this control may be regulated by gibberellic acid (GA), which has stimulated synthesis under blue light conditions and has been shown to be able to inhibit tuberization (Rodríguez-Falcón *et al.*, 2006). An alternative regulation is through StCOL1 stability. Next to red light, Abelenda *et al.* (2016) showed that StCOL1 accumulated under constant blue light as well, making it plausible that cryptochromes play a role in StCOL1 stabilization as well as PHYB (Abelenda *et al.*, 2016). Blue light may also affect tuberization by acting on several proteins in the photoperiodic tuberization pathway. In Arabidopsis, blue light is needed to form the GI-FKF1 complex that degrades CDF and enables *CO* expression (Sawa *et al.*, 2007). Furthermore, in response to blue light, FKF1 binds to CO and increases its stability (Song *et al.,* 2012). Finally, blue light activated cryptochrome suppresses the degradation of CO by the COP1-SPA1 complex (Zuo *et al.*, 2011). Also PHYA was found to inhibit the COP1–SPA1 complex, but the mechanism behind this control is still unknown (Andrés and Coupland, 2012).

Light spectrum may influence tuberization on multiple levels, which opens up possibilities to use light spectrum to control tuberization. How light spectrum influences potato flowering is still unknown. In this thesis we will implement knowledge on light spectral regulation, by using different light spectra to control tuberization and find out how potato flowering is affected.

Daily light integral and light intensity

It is difficult to distinguish between light intensity effects and DLI effects. A light level effect, which encompasses both intensity and DLI, may therefore be regulated in multiple ways. Although a lot is known about the perception of light spectrum by photoreceptors, very little is known about how plants sense light intensity. It has been discovered that photoreceptors like phytochrome are able to sense light intensity as well as spectrum (Li *et al.*, 2011; Trupkin *et al.*, 2014). However, light level may control processes like flowering and tuberization through the formation of assimilates (Thomas, 2006). Altering the amount of light available to the plant, can also alter the total photosynthesis and assimilate production. Carbohydrates have been shown to play a fundamental role in flowering control in many species (Bernier and Périlleux, 2005; Srikanth and Schmid, 2011). For instance, an exogenous supply of carbohydrates to plants that required high light to flower, allowed them to flower under low light conditions (King and Bagnall, 1996; Thomas, 2006). The role of carbohydrates in Arabidopsis flowering has been extensively studied and has revealed a sugar mediated flowering pathway (Bouché *et al.*, 2016). Although flower initiation in the meristem can be directly affected by sugars, the sugar-mediated flowering pathway also acts on the photoperiodic flowering time pathway, by affecting *GI* and *FT* (Bouché *et al.*, 2016).

In potato very little is known about how carbohydrates regulate tuberization and flowering. One of the few studies, by Chincinska *et al.* (2008), uncovered that a sucrose transporter StSUT4 inhibits tuberization and flowering in potato. By silencing this sugar transporter, potato plants flowered earlier and were able to tuberize in non-inducing long-day conditions (Chincinska *et al.*, 2008). *StSUT4 RNAi* plants had a modified sucrose efflux from the leaves which led to a build-up of sucrose in sink tissues. Flowering was preceded by an increase in sucrose in the apical meristem and in *StSUT4 RNAi* plants this increase happened earlier than in wild-type plants (Chincinska *et al.*, 2008).

Although very little information is available on light intensity or DLI regulated potato flowering and tuberization, it is plausible that assimilate localization plays a role in this control. It would be valuable to understand how light quantity affects potato flowering and tuberization and discover whether this control is mediated by assimilates.

Model

The molecular regulation of tuberization and flowering by light is illustrated in Fig. 1.1. Photoperiod, light spectrum, light intensity and the DLI regulate tuberization and flowering through connected pathways.

This thesis

In this thesis I aim to increase the knowledge on the reproduction methods of potato. Not only will both tuberization and flowering be studied in detail, but it will also be determined how both processes are regulated simultaneously, which is often overlooked in tuberization studies. Specifically, I will determine how light spectrum affects tuberization and flowering by applying different LED light spectra. Information on light spectral effects on tuberization is limited and the influence of light spectrum on potato flowering has not yet been studied. I will go deeper into the photoperiodic regulation of tuberization, fine-tuning our information on the mechanisms behind this control and testing whether the well-defined coincidence model in Arabidopsis holds in potato. Also, I will determine whether flower initiation is day neutral, by comparing day lengths without changing the DLI. The DLI effect on flower initiation will be looked into in more detail. Not only initiation time in the number of leaves before the inflorescence will be determined, but also as the time in days before macroscopic flower bud appearance. The mechanisms behind a DLI effect will be studied in detail. Finally, flower bud development will be studied and it will be determined whether this development is affected by competition with the tubers. As it has not yet been verified if competition between flowers and tubers really exists and how this competition may work, it will be determined how flowering is affected when potato plants tuberize.

Many experiments on tuberization and flowering show inconsistencies, mainly due to mixed effects of multiple environmental factors (e.g. DLI effects while comparing photoperiod). Therefore, I will perform experiments with LED lights in a controlled environment. This way climate factors can be separated and regulated, making sure effects on flowering and tuberization are attributed to the environmental factor of interest. Finally, a part of the mechanisms behind light controlled tuberization and flowering will be elucidated, by performing

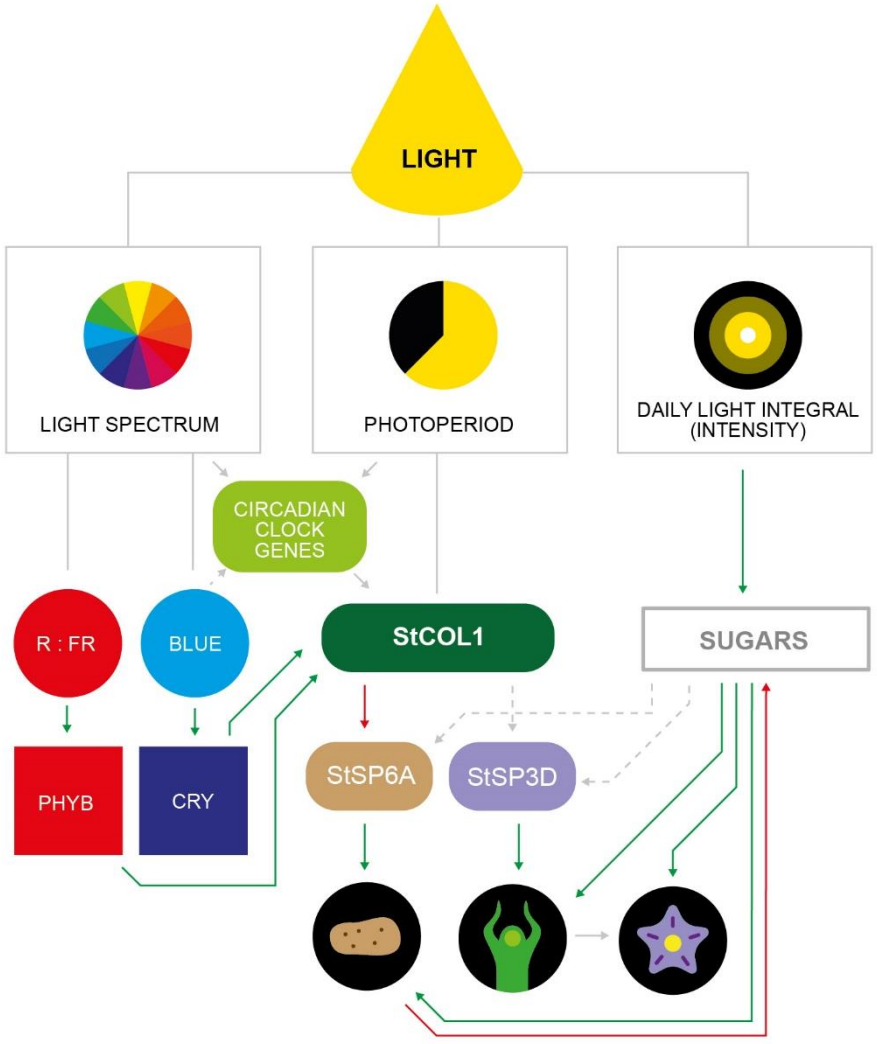


Figure 1.1. A model of light mediated tuberization and flowering control in potato. Light can be divided into three main components. The photoperiodic tuberization pathway involves circadian clock regulated genes, which control potato *CONSTANS (StCOL1)* expression, which in turn represses tuberization by inhibiting the tuberization signal *StSP6A.* The control of StCOL1 on *StSP3D* is not understood, however, StSP3D is thought to be involved in flower transition (in the shoot apical meristem). Light spectrum acts on tuberization through photoreceptors phytochrome B (PHYB) and cryptochrome (CRY), which are controlled by the red/far-red ratio and blue light. Both photoreceptors stabilize StCOL1 protein. Light spectrum may also affect potato reproduction by acting on circadian clock genes. The light level, which may be an effect of the daily light integral, or the light intensity, may regulate flowering and tuberization through assimilate formation. The sugars may control flower and tuber initiation directly (unknown pathway), or through *StSP6A* and *StSP3D* (still unknown). Once induced, tuber and flower bud development (into open flowers), may depend on the amount of sugars available. Finally, tuberization may negatively affect flowering by acting as an assimilate sink and reducing the sugars available for flower development. Green arrows indicate positive effects and red arrows negative effects.

gene expression analyses, assimilate concentration measurements and protein detections.

Objective

The goal of this PhD research is to understand how light regulates both tuberization and flowering in potato. I will do this by:

- Quantifying the effects of photoperiod, light spectrum, light intensity and DLI on tuberization and flowering time.
- Determining if tuberization and flowering compete and if so, how this is regulated.
- Discovering which molecular mechanisms underlie regulation of tuberization and flowering by light.

Once it is understood how light regulates tuberization and flowering, light can be used to create an optimal environment for either tuberization or flowering. For potato production, tuberization can be stimulated, and for potato breeding, flowering can be stimulated.

Thesis outline (Fig. 1.2)

My thesis contains six chapters: this general introduction (Chapter 1), four research chapters (Chapters 2-5) and a general discussion (Chapter 6).

In **Chapter 2** we focus on the influence of light spectrum and photoperiod on potato flowering and tuberization. We demonstrate for the first time, the effect of far-red light on potato tuberization and flowering, when applied throughout the day. Furthermore, the effect of blue light supplemented to regular light is shown on potato reproduction. Finally, we extended day length with low intensity white or blue light to determine the effect of photoperiod on tuberization and flowering, without changing the DLI.

In **Chapter 3** we determine how a night break in potato is controlled. A night break, a short period of light in the middle of the night, can be applied to inhibit tuberization in a short day. However, it is not yet known how this control works. We hypothesize that the coincidence model explaining Arabidopsis flowering, where flowering depends on the expression of *CO* coinciding with light, also controls potato tuberization. In potato a night break may be effective

because the light of the night break coincides with *StCOL1* expression in the night. We determine tuberization in several night-break treatments, in which the light of the night break either coincides with *StCOL1* expression or not.

In **Chapter 4** we demonstrate the effect of DLI on flower initiation time. Flower bud appearance time and leaves formed before the inflorescence are studied under several DLIs. Also we determine whether the DLI effect acts through carbohydrates and the flowering time gene *StSP3D*.

In **Chapter 5** we focus on competition between flowering and tuberization. We determine whether the tuber sink impairs flower development and elucidate which role the tuberization signal StSP6A plays in flower development impairment.

In **Chapter 6** the main findings of this thesis are discussed. I present an overview of the effects of light on potato reproduction and summarize the main mechanisms behind this control. Furthermore, I discuss unresolved questions and briefly give examples for future implementation possibilities for regulating tuberization and flowering with light.

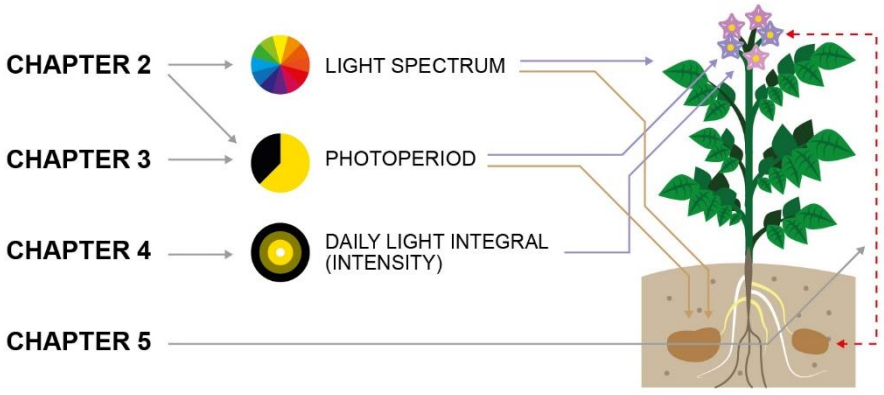


Figure 1.2. The regulation of potato tuberization and flowering by light. Chapter 2 focusses on the effect of light spectrum and photoperiod on flowering and tuberization. Chapter 3 dives deeper into the photoperiodic regulation of tuberization and also briefly mentions the effect on flowering. Chapter 4 discusses the effect of the daily light integral (or perhaps light intensity) on potato flowering. In Chapter 5 it is determined whether tuberization and flowering compete for assimilates.

2. Regulating flower and tuber formation in potato with light spectrum and day length

Faline Plantenga, Melpomeni Siakou, Sara Bergonzi, Ep Heuvelink, Christian Bachem, Richard Visser and Leo Marcelis

Adapted from: Regulating flower and tuber formation in potato with light spectrum and day length, Acta Horticulturae, 2016, 1134, 267-276, DOI: 10.17660/ActaHortic.2016.1134.36

Abstract

Solanum tuberosum (potato) can reproduce through tubers and through seeds. Recent developments have enabled hybrid breeding and propagation from seeds in this crop. This makes potato flowering a new focus of research. Tuberization and presumably flowering followed by seed set are strongly regulated by environmental cues. A well-studied environmental regulator of tuber formation is day length. Photoreceptors are involved in this photoperiodic control of tuberization, suggesting light spectrum may be an important factor for tuberization. However, it is not known how photoreceptors control potato flowering. Here, we aim to elucidate the influence of light spectrum and photoperiod on tuber and flower formation, by growing three potato genotypes in climate chambers with light-emitting diode (LED) lighting and additional far-red and blue LEDs under long and short days. Far-red light accelerated tuber formation up to eleven days and blue light slightly delayed it up to four days. An effect of light spectrum on flowering time was not found. Long photoperiods delayed tuber formation compared to short-day conditions in two of the three tested genotypes. Aside from one genotype which only flowered in long days, no effect of photoperiod on flowering time was found.

Keywords: tuberization, flowering, far-red light, blue light, photoperiod, LED

Introduction

Solanum tuberosum (potato) is the third most consumed crop by humans worldwide (International Potato Center, 2015). The potato plant is also of scientific interest due to its dual reproduction method; it can reproduce asexually through tubers and sexually through seeds. A large volume of research has been done on potato plant development, but in most cases the focus has been on tuberization. Until now, little research has been done on potato flowering, let alone focused on both tuberization and flowering (Almekinders and Struik, 1996; Chincinska *et al.*, 2008; González-Schain and Suárez-López, 2008).

Hybrid crossing of homozygous parents is a breeding method applied in many crops. Commercial potato genotypes are tetraploid, extremely heterozygous and suffer from inbreeding depression when self-fertilized. This makes it problematic to create homozygous potato material and subsequently use hybrid breeding in potato. Diploid potato material is a more practical target, but most diploid potatoes are self-incompatible, making it impossible to produce homozygous material (Hirsch *et al.*, 2013). Recent developments have enabled self-fertilization in diploid potato accessions, leading to increasingly homozygous material with each crossing event and enabling the use of hybrid breeding in potato (Lindhout *et al.*, 2011). This is advantageous for many reasons: selection of desired traits can be done more specifically, the time necessary to make a new cultivar can be decreased considerably, and because hybrid breeding makes use of seeds for propagation instead of tubers, transportation and storage is easier and cheaper. With the increasing importance of potato seeds, interest in potato flowering is growing. Developing ways to regulate either tuberization or flowering in controlled environments could lead to new opportunities. Potato flowering could be specifically induced to speed up the breeding process, while tuberization could be induced when potatoes are desired. Depending on the purpose of the grower, either reproduction method could be stimulated.

Tuber formation is controlled strongly by the environment and by light in particular. Potato plants are induced to tuberize in short days. In the obligate short-day potato *Solanum tuberosum* L. ssp. *andigena* (*S. andigena*) tuberization will only occur in short days, while commercially used genotypes are able to tuberize in long days as well (facultative short day plants) (Batutis and Ewing, 1982; González-Schain *et al.*, 2012). Whether flower induction in potato is a long-

day, short-day or a day length neutral process, is still under discussion (Almekinders and Struik, 1996; Macháčková *et al.*, 1998; González-Schain and Suárez-López, 2008). Photoperiodic control of tuberization works through several genes, which are also involved in flowering in *Arabidopsis thaliana* and are conserved in many species (Tsuji *et al.*, 2011; Andrés and Coupland, 2012; Fu *et al.*, 2014). It can therefore be expected that not only tuberization, but also flower induction in potato is regulated by the same set of genes. The transcription factor *CONSTANS (CO)* is one of the genes responsible for photoperiodic flowering control in Arabidopsis. A homolog of this gene, called *StCOL1* in potato, regulates tuberization (González-Schain *et al.*, 2012). Long days in *S. andigena* stabilize StCOL1 protein, inhibiting the tuberization signal. In short days, StCOL1 activity is suppressed, enabling tuber formation. Photoreceptors can relay signals from the environment to the plant and phytochrome B (PHYB) has been found to be involved in photoperiod dependent tuber formation (Jackson *et al.*, 1996). PHYB is thought to stabilize StCOL1 and thereby inhibit tuber formation in long days (Navarro *et al.*, 2011; Navarro *et al.*, 2015). Transgenic plants lacking PHYB tuberize independent of day length in short and long days (Jackson *et al.*, 1996).

PHYB activity is affected by light spectrum; red light activates PHYB (P_{fr}), while far-red light inactivates PHYB (P_r) (Casal, 2013). Next to red and far-red light, blue light has been found to affect tuberization (Fixen *et al.*, 2011). Flowering in Arabidopsis is partially regulated by the blue light photoreceptor cryptochrome (CRY); in long days, blue light activates CRY at the appropriate time to enable CO activity (Andrés and Coupland, 2012). Whether blue light can inhibit potato tuberization through stabilization of StCOL1 is unknown. Furthermore, it is not clear how StCOL1 influences potato flowering.

To test how potato flowering and tuberization are controlled by photoperiod and light spectrum, three genotypes were grown under white light or white light with additional far-red or blue light. Far-red light was used to initiate tuberization in long days, through the inactivation of PHYB. Blue light was used to inhibit tuberization in short days, through the activation of CRY and stabilization of the StCOL1 protein. Furthermore the effect of both far-red and blue light on potato flowering was determined. It was verified if the actual day length affected tuberization and not the higher amount of light energy acquired in longer days compared to short days. Also, it was elucidated whether potato flowering is a short-day, day length neutral or long-day process. The effect of day

length on tuberization and flowering was tested by extending short days with a low level of light that could be sensed by the plant as a long day but would not significantly add to the total amount of acquired light energy.

Materials and methods

Plant material and growing conditions

Three genotypes were used: an obligate short-day tuberizing tetraploid *Solanum tuberosum* L. ssp. a*ndigena*, and two diploids RH 89-39-16 (RH) and G254 (Hermsen, *et al.*, 1978; Potato Genome Sequencing Consortium, 2011). The three genotypes were propagated *in vitro*. After three multiplication rounds, the *in vitro* plantlets were transferred to soil in 17cm pots filled with a mixture of clay-peat potting soil (5.7 pH, 0.8 dS m^{-1}) and placed in two climate chambers, one for the far-red experiment in long days and one for the blue light experiment in short and extended days. The relative humidity in both chambers was set to 80% and the temperature to 20°C. Water was given manually and liquid fertilizer (Hydro, Substrafeed™ Pakket El: 35.1 % N, 40.4 % S, 42.5 % P, 35.8 % K, 17.5 % CaO with an EC of 2.0) was applied to the plants once a week. Initially the plants were grown under white/red LEDs (Philips GreenPower LED production module 120cm DeepRedWhite-2012, where white LEDs are phosphor coated blue-dyed LEDs) with a photosynthetic photon flux density (PPFD) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 16 hours per day for two weeks, before starting the treatments. All side shoots were removed.

Experimental set-up

Both climate chambers were divided into four compartments, each containing a light treatment. Ten plants per genotype were divided evenly across each compartment and within compartments pots were rotated three times a week to ensure plants received the same amount of light during the experiment. The long-day far-red light climate chamber received 16 hours of light, while the short-day blue light chamber received 8 hours of light (and two treatments received additional low intensity light). The far-red experiment consisted of three long-day far-red treatments and a treatment without far red and the blue

light experiment consisted of a short-day white light treatment, a short-day blue + white light treatment and two extended day treatments with either white or blue light. In all treatments the standard "white" light source consisted of white/red LEDs with, depending on the treatment, additional far-red, blue or white LEDs (Philips GreenPower LED, far red: production module 120cm far red and research module far red, blue light: research module blue, additional white light: research module white). The light intensity (PPFD) perceived at the top of the canopy in each treatment was 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and was measured with a quantum sensor (LI-COR Biosciences, LI-1400 Datalogger) and corrected by adjusting LED height every two weeks. As the wavelength of far-red light (in this experiment 700-765nm) lies beyond the photosynthetic active radiation (400-700nm), adding far-red light did not increase the total PPFD of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Table 2.1). The light spectrum was measured with a spectroradiometer (USB2000 spectrometer, Ocean Optics, Duiven, The Netherlands). The phytochrome stationary state (PSS), the ratio of active PHYB P_{fr} compared to the total active and inactive PHYB, was calculated from the light spectrum, as described by Sager *et al.* (1988) (Table 2.1).

Measurements

To determine flowering and tuber formation time, plants were checked for any signs of flower buds or tubers twice a week by checking the shoot apical

Table 2.1. Light characteristics per treatment in the far-red (FR) and blue (B) light experiments.

Experiment	Treatment name	Light conditions	PSS[1]-value
FR in long days	No FR	16h W[2] (200[3])	0.88
	Medium FR	16h W (200) + FR (50)	0.82
	High FR	16h W (200) + FR (100)	0.75
	EOD[4] FR	16h W (200) **followed by 25min** FR (10)	0.88+**0.10**
B in short and extended days	W	8h W (200)	0.88
	W + B	8h W (100) + B (100)	0.85
	Extended W	8h W (200) **followed by 8h** W (5)	0.88+**0.84**
	Extended B	8h W (200) **followed by 8h** B (5)	0.88+**0.46**

[1]PSS = phytochrome stationary states. "1" is only P_{fr} and "0" is no P_{fr}.
[2]W = white light.
[3]Light intensities in brackets were measured in $\mu mol \cdot m^{-2} \cdot s^{-1}$.
[4]EOD = end-of-day.

meristem visually and by carefully removing the soil around the stem and stolons and checking for swelling of the stolon tip. Twice a week plant height (from the first compound leaf to the top of the plant) and the number of compound leaves were determined of all plants. After six weeks destructive measurements were performed. These included plant height, leaf number, total leaf area measured with a leaf area meter (LI-COR Biosciences, LI-3100C area meter), the number of tubers and the fresh and dry weight of the shoot and tubers.

Data analysis

Statistical analyses were performed using GenStat 17th Edition. The three cultivars were analyzed separately using a one-way analysis of variance. The effect of light treatments on tuberization and flowering time and plant morphology was determined. Significance was tested with a *P*-value <0.05. Multiple comparisons were made using the Bonferroni procedure. There were no real repetitions in this experiment due to the confined space of the climate chambers; every plant was considered as an independent experimental unit.

Results

Far-red light

In long days tuber appearance of genotypes RH and G254 was accelerated by far-red light (Fig. 2.1A). This effect was significant in all far-red treatments in G254 and in the treatments with end-of-day and high intensity far-red light in RH. Obligate short-day *S. andigena* did not tuberize in any of the treatments. Flower buds formed in all genotypes, but the time until flower bud formation was not significantly different between treatments (Fig. 2.1B).

Morphological differences were found between light treatments in the far-red experiment (Table 2.2.). Adding far-red light throughout the day increased plant height; the effect was largest for the high far-red intensity. Although end-of-day far-red light significantly increased plant height in RH and G254, these effects were smaller than that of the high and medium far-red light treatment. In *S. andigena* and G254, the leaf area was not significantly changed by the far-red treatments. In RH only the medium far-red treatment had a significantly lower

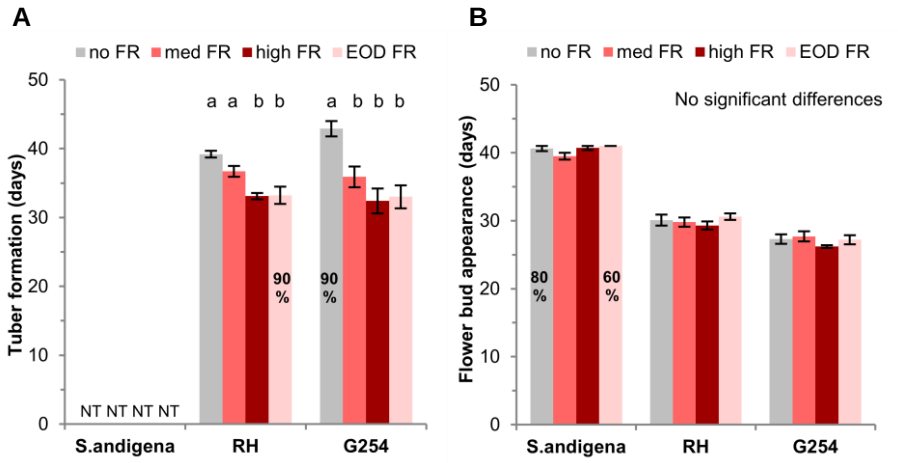


Figure 2.1. Far-red light experiment. Tuber (A) and flower bud (B) appearance time in response to far-red (FR) light in long days for three genotypes (*S. andigena*, RH and G254). Time was counted from transplanting. "NT" = no tuberization after six weeks. No FR = white light, Med FR = white + medium intensity FR light, High FR = white + high intensity FR light, and EOD FR = white + end-of-day FR light. If some plants failed to tuberize or flower after six weeks, the percentage of plants that did flower or tuberize is given. Treatments within a genotype, with the same letter were not significantly different.

leaf area. Although not significant, the differences in leaf area in G254 could be caused by intumescence in the leaves, which was found in all long-day treatments, with the exception of the EOD far-red light treatment. Treatments had little effect on the number of leaves. Most treatments had slightly less leaves in the far-red treatments, but this effect was not significant in all treatments. The number of tubers was not significantly different between treatments, with the exception of G254, which produced more tubers under medium far-red light. Tuber biomass was higher in plants exposed to far-red light, albeit not significantly higher in all far-red treatments. For the shoot dry weight inconsistent results were found. RH showed no difference between treatments, *S. andigena* had a lower shoot biomass in the EOD far-red treatment and G254 had a higher shoot biomass in the high far-red treatment.

Blue light

Tuberization in blue light was only significantly delayed in G254 (Fig. 2.2A). In *S. andigena* and G254 the extended white light treatment led to an inhibition of

Table 2.2. Morphological traits of three potato genotypes in the far-red (FR) and blue (B) light experiment.

			Height (cm)	Leaf area (cm^2)	Leaves (#)	Tubers (#)	Shoot DW[1] (g)	Tuber DW (g)
S. andigena	FR in long days	no FR	23.6 c[2]	974 a	20.1 a	0.0 -	5.7 ab	- -
		mediumFR	42.6 b	812 a	19.1 b	0.0 -	5.5 b	- -
		high FR	61.0 a	774 a	19.4 ab	0.0 -	6.0 ab	- -
		EOD[3] FR	27.2 c	1418 a	19.1 b	0.0 -	4.9 c	- -
	B in short and extended days	W[4]	24.6 b	644 a	15.4 b	3.5 a	1.7 bc	0.4 a
		W + B	7.6 c	669 a	14.9 b	2.9 a	1.6 c	0.1 b
		Extended W	28.5 b	772 a	17.8 a	0.0 b	2.5 a	- -
		Extended B	41.9 a	655 a	18.1 a	0.9 b	2.1 ab	0.1 b
RH	FR in long days	no FR	15.9 d	1479 a	15.8 a	18.6 a	6.1 a	2.2 c
		mediumFR	36.7 b	1261 b	14.7 a	17.5 a	6.2 a	5.1 b
		high FR	53.1 a	1310 ab	15.1 a	21.6 a	6. 5 a	7.2 a
		EOD FR	28.5 c	1327 ab	14.9 a	16.8 a	6.4 a	3.8 bc
	B in short and extended days	W	16.3 b	1511 a	12.5 a	16.9 ab	3.8 b	3.7 a
		W + B	8.0 c	1227 a	12.0 a	15.1 b	3.4 b	1.8 b
		Extended W	17.5 b	1549 a	12.2 a	21.3 a	4.7 a	2.7 ab
		Extended B	27.6 a	1344 a	12.4 a	20.8 ab	4.0 ab	3.8 a
G254	FR in long days	no FR	16.4 d	895 ab	14.5 a	4.2 b	4.8 b	0.2 b
		mediumFR	38.4 b	783 b	13.0 b	7.3 a	5.3 b	0.9 ab
		high FR	59.3 a	868 b	13.0 b	7.1 ab	6.4 a	1.3 a
		EOD FR	20.6 c	994 a	13.4 ab	4.5 ab	5.0 b	1.7 a
	B in short and extended days	W	15.8 b	1274 a	12.5 a	15.5 a	3.5 b	0.7 a
		W + B	8.1 c	1016 a	12.1 a	13.3 a	3.1 b	0.1 b
		Extended W	16.1 b	1036 a	11.9 a	0.0 b	3.5 b	- -
		Extended B	33.7 a	1294 a	12.0 a	20.2 a	4.6 a	0.5 ab

[1]DW = dry weight.
[2]Same letters indicate no significant difference between treatments within an experiment.
[3]EOD = end-of-day.
[4]W = white light.

tuberization. The extension with blue light only affected *S. andigena*; only 20% of the plants formed tubers and formation time was delayed. In RH, treatments had no effect on tuberization time. Flowering was not affected by the treatments, with the exception of *S. andigena* in the extended white light treatment. *S. andigena* only formed visible flower buds in this treatment (Fig. 2.2B).

There were clear morphological differences between the treatments of the blue light experiment. In short days with blue light, plants of all three genotypes remained extremely short, but when the day length was extended with blue light the plants were significantly taller than in other treatments (Table

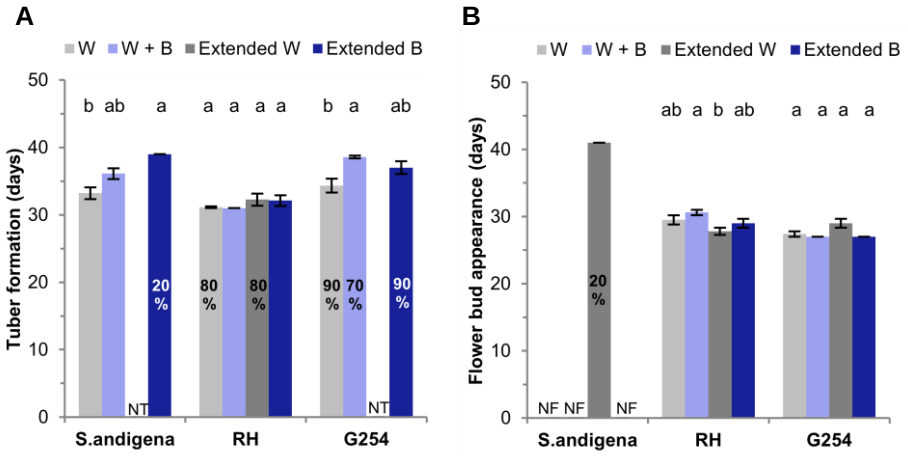


Figure 2.2. Blue light experiment. Tuber (A) and flower bud (B) appearance time in response to blue light in short days and extended days with white or blue light for three genotypes (*S. andigena*, RH and G254). Time was counted from transplanting. W = short day white light, W+B = short day white + blue light, Extended W = short day extended with white light and Extended B = short day extended with blue light. "NT" = no tuberization after six weeks. "NF" = no flowering after six weeks. If some plants failed to tuberize or flower after six weeks, the percentage of plants that did flower or tuberize is given. Treatments within a genotype, with the same letter were not significantly different.

2.2.). There was no significant difference in height between the short-day white light treatment and the extension with white light. The total leaf area was similar between treatments, as was the leaf number, except for *S. andigena* where the plants with extended day length contained more leaves. The extension of day length with white light led to no tubers in *S. andigena* and G254. Extension with blue light led to a smaller tuber number in *S. andigena* and in G254 the tuber number was comparable to that of the short-day white light treatment. Light treatments had no significant effect on the tuber number in RH. The biomass between treatments differed slightly. Extended white light increased shoot biomass in *S. andigena* and RH, compared to short days with white light, but in G254 only extension with blue light led to a higher biomass. Tuber biomass in blue light was decreased in all genotypes. Extension with white light eliminated tuber formation in *S. andigena* and G254, while it significantly decreased tuber biomass in RH. The extension with blue light only caused a decrease in tuber biomass in *S. andigena.*

Discussion

Far-red light

Although the genotypes RH and G254 could tuberize in long days, tuberization was delayed when compared to short days (blue light experiment). Addition of far-red light in long days, accelerated tuberization to a short day level. However, far-red light did not initiate tuberization in non-induced plants (*S. andigena*). Transgenic *S. andigena*, unable to express PHYB, tuberizes in long days (Jackson *et al.*, 1996), implying inactivation of PHYB with far-red light is not as effective as no PHYB at all. This is not surprising considering the PSS values of the high and medium far-red treatments (0.75 and 0.82 respectively), which indicate that PHYB is still partially active. This is also the case for the EOD treatment which has a low PSS at the end of the day (0.10) but a high PSS throughout the day (0.88). It seems that even with the decreased active PHYB, enough StCOL1 in *S. andigena* is stabilized to inhibit tuberization. RH and G254 tuberized later in long days compared to short days, suggesting some stable StCOL1 was present in the long day treatments. Perhaps the decreased PHYB activity was sufficient to prevent stabilization of low levels of StCOL1, leading to acceleration of tuberization in these genotypes. Furthermore, the EOD far-red treatment, led to the same accelerated tuberization as the high far-red treatment, despite the short duration of time the EOD far-red light was given. This indicates that potato tuberization is sensitive to PHYB activity at the end of the day. Flowering time was not influenced by the far-red light treatments, which suggests that flowering in potato is not regulated through PHYB.

Far-red light increased plant height while the number of leaves hardly changed, indicating internode elongation was stimulated. This corresponds with the shade-avoidance syndrome found in plants where a lowering of the red/far-red ratio leads to plant elongation (Casal, 2013). The end-of-day treatment had much less effect on elongation than the medium far-red treatment. Therefore, unlike tuberization, plant elongation seems to be more sensitive to small changes in the red/far-red ratio throughout the day compared to the end of the day. This timing of and sensitivity to signals controlling plant processes ensures activation at an optimal moment in the day (de Montaigu *et al.*, 2015). Differences in tuber biomass between treatments were not consistent between genotypes. In G254 tuber biomass was higher in plants that tuberized earlier, suggesting that early

tuberization allows more time to load assimilates into the tubers. RH plants with medium and high far-red light, had a higher tuber biomass, corresponding more to the elongation response than to the tuberization time. Elongated plants have a more open structure which allows for better light sequestration and higher rate of assimilate production (Sarlikioti *et al.*, 2011), resulting in more assimilates which can be invested in the tubers. Finally, severe intumescence occurred in the leaves of G254 plants. This leaf disorder has been found in controlled environments and its cause remains elusive (Craver, Miller, Williams, & Bello, 2014)(Craver, *et al.*, 2014). Previous research has discovered that application with UV lighting can diminish intumescence damage (Craver *et al.*, 2014). In the far-red and blue light experiments, only long days resulted in serious intumescence, while short days, which had the same light quality, and extended days, which had the same day length, only showed minor cases of intumescence. Therefore a probable cause of intumescence is the total daily light quantity (11.52 $mol \cdot m^{-2} \cdot d^{-1}$ in the long days compared to 5.76 $mol \cdot m^{-2} \cdot d^{-1}$ short days). This research also demonstrated that end-of-day far-red light minimized intumescence damage in potato.

Blue light

The tuberization time of G254 was delayed in short days with blue light. This might have been related to the fact that the growth of the plants was stunted. Extending days with white light fully inhibited tuberization in *S. andigena* and G254, proving long days and not a higher light quantity inhibits tuberization in these genotypes. Extension with blue light had a less severe effect; tuberization in G254 was not delayed but in *S. andigena* only 20% of the plants managed to tuberize and this was later than in short days under white light. So extending day length with blue light seems less effective than extending it with white light. This makes sense as the red wavelengths, which are present in the white but not in the blue light extension, can activate PHYB and stabilize StCOL1 and thereby inhibit tuberization. In short days blue light did not affect flowering. The extension with white or blue light did show different flowering times in *S. andigena,* which only had visible flower buds in the extended day with white light. Maybe potato flowering in this genotype is induced by long days, as was stated in Macháčková *et al.* (1998), and only extension with white ligth is sensed as a long day. Or perhaps *S. andigena* flowering only occurred in the extended

white light treatment because it coincided with the absence of tuberization. However, in G254 and RH, flowering and tuberization took place at the same time in short and long days, indicating both processes can happen simultaneously and that flowering is day neutral in these genotypes as proposed by Almekinders and Struik (1996).

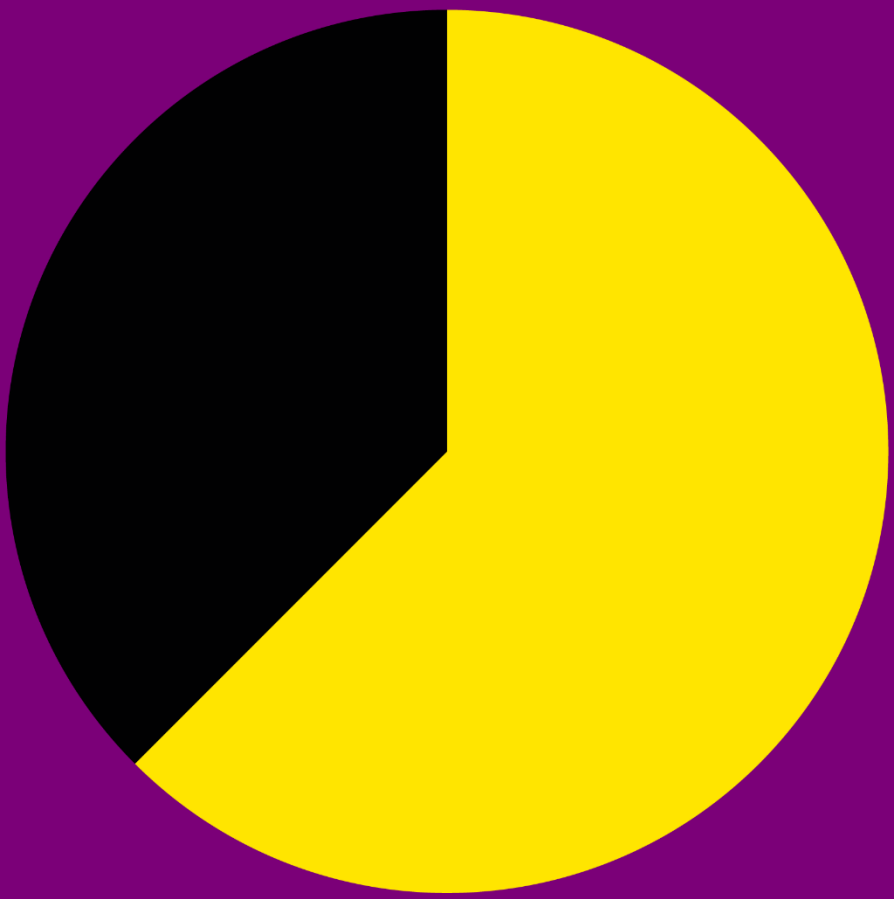
Blue light in combination with white light led to short plants, but blue light given without the presence of other wavelengths led to strongly elongated plants. PHYB, partially inactivated by far-red light, results in plant elongation, but blue light can also convert PHYB to its inactive state, as is apparent when observing the PSS value of the extended day treatment with blue light (0.46). The low PHYB activity in this treatment explains the observed elongation. The PSS value of the white with blue light treatment is similar to that of a white light treatment (0.85), explaining why plants in this treatment were not elongated.

Conclusion

This research shows that by altering the light spectrum only, it is possible to accelerate and delay tuberization in potato. However, this does not affect all tested genotypes to the same extent. Application of far-red light leads to the acceleration of tuberization in long days, but is not capable of inducing tuberization in obligate short-day potatoes. Far-red light is effective when applied at high intensities during day time as well as at a low intensity for a short time at the end of the day. Potato flowering is not influenced by far-red light. Blue light slightly delays tuberization in some genotypes but does not affect potato flowering. Different day lengths do not influence potato flowering, with the exception of *S. andigena* which only had visible flower buds in long days, which coincides with the absence of tubers.

Acknowledgments

This research was supported by the Netherlands Organization for Scientific Research (NWO), Solynta, Signify (Philips), Incotec and Beekenkamp.



3. Coincidence of potato *CONSTANS (StCOL1)* expression and light cannot explain night-break inhibition of tuberization

Faline Plantenga, Ep Heuvelink, Juriaan Rienstra, Richard Visser, Christian Bachem and Leo Marcelis

A revised version was accepted for publication in Physiologia Plantarum, 2018, DOI: 10.1111/ppl.12885

Abstract

In the obligate short-day potato *Solanum tuberosum* L. ssp. *andigena,* short days, or actually long nights, induce tuberization. Applying a night break in the middle of this long night inhibits tuberization. However, it is not yet understood how this inhibition takes place. We suggest a coincidence model, similar to the model explaining photoperiodic flowering in Arabidopsis. We hypothesize that potato *CONSTANS* (*StCOL1),* expressed in the night of a short day, is stabilized by the light of the night break. This allows for StCOL1 to inhibit tuberization through induction of *StSP5G,* which represses the tuberization signal *StSP6A*. We grew *S. andigena* plants in short days, with night breaks applied at different time points during the dark period, either coinciding with *StCOL1* expression or not. StCOL1 protein presence, *StCOL1* expression and expression of downstream targets *StSP5G* and *StSP6A* were measured during a 24-hour time course. Our results show that a night break applied during peak *StCOL1* expression is unable to inhibit tuberization, while coincidence with low or no *StCOL1* expression leads to inhibited or severely repressed tuberization. These results imply that coincidence between *StCOL1* expression and light does not explain why a night break inhibits tuberization in short days. Furthermore, stable StCOL1 did not always induce *StSP5G,* and upregulated *StSP5G* did not always fully repress *StSP6A*. Our findings suggest there is a yet unknown level of control between *StCOL1, StSP5G* and *StSP6A* expression, which determines whether or not a plant tuberizes.

Keywords: potato tuberization, night break, CONSTANS, StCOL1, StSP5G, StSP6A

Introduction

Globally, potato (*Solanum tuberosum*) is the third most consumed food crop by humans (International Potato Center, 2018). Gaining extensive understanding on tuberization (tuber formation) can give growers the tools that are needed to optimize growth and tuber yield.

Light strongly influences potato plant development and the day length is a key factor controlling tuberization (Prat, 2010). Shorter day lengths promote tuberization in potatoes (Garner and Allard, 1923, Rodríguez-Falcón *et al.,* 2006). Although tuberization in all potato varieties is induced in short days, not all varieties require short days to tuberize (Driver and Hawkes, 1943). *Solanum tuberosum* L. ssp. *andigena* (*S. andigena*), is a variety that originated in the Andes, and is used as a model potato plant for photoperiod studies, because of its strict short-day requirement for tuberization (<12 hours light) (Ewing and Struik, 1992, Jackson, 1999, Hannapel, 2007). It is supposed that not the length of the light period, but the length of the dark period is critical for tuberization (Jackson, 1999), therefore not short days but long nights are inducing for tuberization. A night break, i.e. a short period of white or red light in the middle of the night, can be applied during a long night to inhibit tuberization (Batutis and Ewing, 1982, Jackson *et al.,* 1996, Macháčková *et al.,* 1998). A night break not only inhibits short-day tuberization, but also short-day flowering in species like Chrysanthemum (Borthwick and Cathey, 1962, Horridge and Cockshull, 1989, Higuchi *et al.,* 2012). Furthermore, night breaks given in the long nights of a short day can induce flowering in long-day plants like wheat (Pearce *et al.,* 2017). Although a lot of research has been done on night breaks, it is not fully understood how a night break regulates tuberization. It has been suggested that tuberization is inhibited by a night break because the night break divides a long night into two short nights (Jackson, 1999). It also has been proposed that the red/far-red photoreceptor phytochrome B (PHYB) plays a role in the control of a night break. Applying a period of red light in the middle of a long night and subsequently applying far-red light, reversed inhibition on tuberization imposed by a night break (Batutis and Ewing, 1982). However, when these experiments were performed, the molecular control behind tuberization was not yet known. With the help of new molecular knowledge of tuberization regulation, we may be able to unravel the functioning of a night break.

The genes controlling tuberization are conserved even in species that do not form tubers, including *Arabidopsis thaliana, Chrysanthemum lavandulifolium* and *Oryza sativa* (rice). Homologs of these genes are responsible for photoperiodic control of flowering (Martínez-García *et al.,* 2002, Tsuji *et al.,* 2011, Andrés and Coupland, 2012, Fu *et al.,* 2014). In Arabidopsis, long-day flowering is induced through the coincidence of gene expression and light, which only allow induction of *FLOWERING LOCUS T* (*FT*) under long-day conditions (Andrés and Coupland, 2012). *FT* encodes for the mobile florigen which moves from the leaves to the shoot apical meristem where it induces flowering. The molecular control of photoperiodic flowering is reviewed in Imaizumi and Kay (2006), Andrés and Coupland (2012) and Song *et al.* (2014): *CONSTANS* (*CO*) expression is controlled by circadian clock components which at the end of a long day form a complex that degrades an inhibitor of *CO,* allowing for *CO* expression at the end of a long day. In short days, the inhibitor of *CO* is not degraded by this complex, only allowing *CO* to be expressed in the night. Post-translational control of CO by light further determines whether flowering is induced. In the light, photoreceptors Phytochrome A (PHYA; red/far-red light) and Cryptochrome (CRY; blue light) reduce the activity of a complex that degrades CO. In short days *CO* is only expressed in the night where CO is degraded. In long days, *CO* expression takes place in the light where CO is not degraded, leading to the induction of *FT* and the initiation of flowering.

Potato tuberization shares a similar control as flowering in *Arabidopsis*, although short days induce tuberization as opposed to flower induction in long days. In long days, a potato homolog of CO (PGSC0003DMT400026065, named StCONSTANS-like1, StCOL1, in Abelenda *et al.* 2016) induces an *FT*-like repressor *SELF-PRUNING 5G* (*StSP5G*). StSP5G represses the *FT*-like tuberization factor *SELF-PRUNING 6A* (*StSP6A*) (Navarro *et al.,* 2011, González-Schain *et al.,* 2012, Abelenda *et al.,* 2016). In short days, little to no StCOL1 is present to induce *StSP5G*, allowing *StSP6A* expression in the leaves, after which StSP6A travels down to the stolons and induces tuberization (Navarro *et al.,* 2011, Abelenda *et al.,* 2016). Surprisingly, in both long and short days, *StCOL1* is expressed at the end of the night and at the beginning of the day (González-Schain *et al.,* 2012, Abelenda *et al.,* 2016). Why the StCOL1 protein is present in long days and not in short days is not fully understood. Abelenda *et al.* (2016) proposed StCOL1 is only present in long days, because peak *StCOL1* expression coincides with the light in long days, while peak *StCOL1* expression in short days is shifted back and

occurs at the end of the dark period. Song *et al.* (2014) also suggested that occurrence of peak expression in light is crucial for the coincidence model. In long days, coincidence between *StCOL1* peak expression and light would stabilize StCOL1 and inhibit tuberization, while in short days, *StCOL1* peak expression would coincide with the dark and StCOL1 would be degraded.

As is the case in *Arabidopsis,* the stabilization of StCOL1 in potato is also controlled by photoreceptors. In potato, PHYB is responsible for stabilization of StCOL1 in the light (Abelenda *et al.,* 2016). PHYB exists in an active and inactive form; red light (660 nm) activates PHYB and far-red light (730 nm) inactivates it (Casal, 2013). In day light, which contains more red than far-red light, the active form of PHYB is more prevalent. Red and far-red light conversion to the active and inactive forms happens almost instantaneously. Active PHYB can also relax into inactive PHYB in the absence of light, but this dark reversion is a relatively slow process (Fankhauser, 2001, Medzihradszky *et al.,* 2013).

This knowledge on the coincidence model in potato tuberization and involvement of PHYB may explain the molecular basis of a night break. PHYB regulated stabilization of StCOL1 in the light of a night break may be the cause of inhibition of tuberization in short days. However, *StCOL1* expression in a short day only peaks at the end of the night, and is low in the middle of the night when a night break is generally applied (González-Schain *et al.,* 2012). This would make StCOL1 stabilization in the light of a night break impossible. However, relaxation of PHYB to its inactive form may take hours (Ruddat *et al.,* 1997, Fankhauser, 2001). Therefore PHYB may be activated in the light of a night break and remain active long enough to stabilize StCOL1 when *StCOL1* expression peaks at the end of the night. We hypothesize that the light applied during a night break inhibits tuberization by stabilizing StCOL1. StCOL1 can then induce *StSP5G* which represses *StSP6A* and inhibits tuberization.

We aim to discover if night-break inhibited tuberization can be explained by light mediated stabilization of StCOL1 in the night. To find out whether this is the case we grew *S. andigena* plants in short days with night breaks, which were applied at different moments during the night and determined if tuberization was inhibited or not. The night breaks coincided with different levels of *StCOL1* expression, which was measured during a 24-hour time course. Also StCOL1 protein presence was measured, to see when StCOL1 was degraded or stabilized in the night. Furthermore the expression of *StSP5G* and *StSP6A* was measured, to

determine the effect of the different night breaks on down-stream targets of StCOL1.

Materials and methods

Plant materials and growth conditions

A tetraploid, short-day tuberizing *Solanum tuberosum* L. ssp. *andigena* (*S. andigena*) was used. A wild type *S. andigena* was used for scoring tuberization and flowering time and for analyzing gene expression. A *S. andigena* transgenic line overexpressing *StCOL1* with an HA-tag (35S::StCOL1-HA) was used for later protein detection by Western Blot. The transformation was done as described in Navarro *et al.* (2011) and the cloning of the genes and promotors was done as described in Abelenda *et al.* (2016).

The plants were propagated in vitro on MS20 medium (Murashige and Skoog, 1962) and grown at 24°C in long days (16/8 hours light/dark) in fluorescent light with a photosynthetic photon flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The plantlets from tissue culture were transplanted to square pots (7 x 7 x 8 cm) which were filled with a clay-peat mixture and placed in two climate chambers. All plants were watered manually and liquid fertilizer was given once per week (Hydro Substrafeed™: 1.2 mM NH_4^+, 7.2 mM K^+, 4.0 mM Ca^{2+}, 1.82 mM Mg^{2+}, 12.4 mM NO_3^-, 3.32 mM SO_4^{2-}, 10 mM P, 35 µM Fe^{3+}, 8.0 µM Mn^{2+}, 5.0 µM Zn^{2+}, 20 µM B, 0.5 µM Cu^{2+}, 0.5 µM MoO_4^{2-}, with an EC (electrical conductivity) of 2.0 $dS \cdot m^{-1}$ and pH of 5.5). The climate chambers were set at 20ºC day and night, with a relative humidity of 70%. The plants were illuminated by red and white LEDs (light emitting diodes) (GreenPower LED production module 120 cm DeepRedWhite-2012, PSS: 0.88 (phytochrome stationary state; "1" refers to only active PHYB and "0" refers to only inactive PHYB), Philips, Eindhoven, the Netherlands). LED height was adjusted every two weeks to maintain the required intensity (see experimental set-up). The light intensity was measured at the top of the plant canopy with a quantum sensor (LI-190SB quantum sensor, LI-1400 Datalogger, LI-COR Biosciences, Lincoln, NE). Plants were rotated three times a week to ensure uniform light distribution and all side shoots were removed from the plants.

Experimental set-up and measurements

Plants were grown in five climate-chamber compartments, each containing a different light treatment. Light treatments were: 1. a short-day control treatment with 8/16 hours light/dark, 2. a short day with a 30-minute night break at the beginning of the night (ZT(12); Zeitgeber time = hours after start of light period), 3. a short day with a 30-minute night break in the middle of the dark period (ZT(16)), 4. a short day with a 30-minute night break at the end of the night period (ZT(20)), 5. a long-day control treatment with 16/8 hours light/dark. The night breaks were given every night, from the moment the plants were placed in the climate chamber until harvest. The treatments were divided over two climate chambers; one climate chamber was divided into four compartments with short-day treatments and the other climate chamber contained the long-day treatment. 33 plantlets of the wild type and 33 plantlets of the 35S::StCOL1-HA line were placed in each light compartment. The light intensity applied was 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (short day), 282 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (short day + night-break treatments) or 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (long day) (Fig. 3.1). In the night-break treatments the light intensity applied during the light period and during the night breaks was the same. The daily light integral (DLI) of each treatment was the same (8.64 $mol \cdot m^{-2} \cdot d^{-1}$). Four weeks after transplanting, plants were sampled for gene expression analysis and protein detection at eleven time points during a 24-hour cycle (Fig. 3.1). The fifth

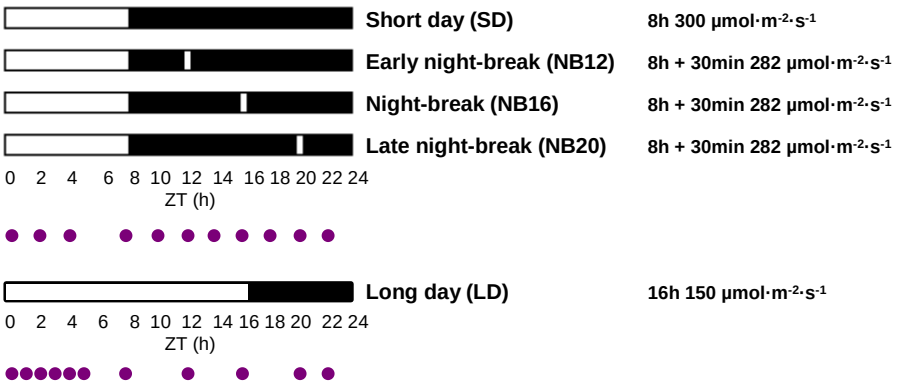


Figure 3.1. Schematic overview of light treatments with or without a night break. *S. andigena* potato plants were grown in five LED light treatments. A short day, three short days with a night break and one long day. The white bar indicates light and the black bar indicates darkness. All treatments received the same DLI (light intensity x hours of light per day). ZT = Zeitgeber time (hours after light stimulation). The dots show time points at which leaf material was sampled for gene expression analysis and protein detection.

leaf from the shoot apex was sampled. Three plants were sampled per time point per treatment and each sample contained two leaflets. Samples were directly frozen in liquid nitrogen and stored at -80ºC. After sampling, ten plants were re-potted to 17 cm Ø pots and maintained for four weeks. During this time, plants were examined for tuberization and flower bud appearance, three times a week (biological replicates n = 10). Destructive measurements were done after eight weeks and included dry weights of stem, leaf, and tubers, and the tuber number.

Gene expression and protein detection

Gene expression was determined using quantitative reverse transcription PCR (RT-qPCR). For RNA extraction, the frozen plant material was ground to powder with a ball mill. Approximately 25 mg of plant sample was placed in a tube, to which 1.2 ml of TRIzol was added (TRIzol: 38% phenol, 0.8 M Guanide Thiocyanate, 0.4 M Ammonium Thiocyanate, 0.1 M NaAc, 5% glycerol, pH 5). After mixing and incubating at room temperature (RT) for 5 min, samples were centrifuged at maximum speed at RT in a table-top centrifuge. 1 ml of the supernatant was transferred, to which 250 µl chloroform was added and shaken. After centrifugation, 450 µl of the aqueous phase was mixed with 1 volume of isopropanol and samples were incubated at RT for 20 min. After centrifugation, the pellet was washed with 70% ethanol and dissolved in Milli-Q water. Several RNA quality steps were performed (Taylor, 2010); the integrity of the RNA was inspected by gel electrophoresis and quality of the RNA was tested by spectrophotometry (NanoDrop, DS11, DeNovix, Wilmington, DE). RNA integrity was checked on agarose gel, for all samples clear rRNA bands were visible (data not shown). 1 µg of RNA was used for DNase treatment using RQ1 RNase-Free DNase (M6101, Promega, Madison, WI). Absence of DNA was verified via qPCR with *ACTIN* primers (see below) on DNase-treated RNA samples (-RT control). The purified RNA was synthesized to cDNA using a high capacity cDNA reverse transcription kit (Thermofisher, Waltham, MA). DNase treatment and cDNA synthesis were performed as per manufacturers' instructions. 20 µl of cDNA was diluted with Milli-Q water to a total volume of 200 µl. The qPCR mix contained 5µl SYBR-green (iQ-SYBR-green Supermix, Bio-Rad, Hercules, CA), 0.5 µl Forward Primer (10 µM), 0.5 µl Reverse Primer (10 µM), 1.5 µl Milli-Q water and 2.5 µl cDNA. The qPCR was performed with a following program in a Thermal Cycler (C1000, Bio-Rad, Hercules, CA): 95°C for 3 min, then 40 cycles alternating

between 95°C for 10 sec and 58°C for 30 sec. The qPCR was followed by 95°C for 10 sec and a melt curve analysis from 65°C to 95°C with 0.5°C increments, each step 10 sec. Three biological replicates were used per time point in each treatment and a negative control (no cDNA) was done per primer pair. The primers used for *StCOL1* (PGSC003DMT400026065) were (F) GTAGCAACAATTGGGCAAGGG, (R) AGTAAACGGTACATGTTGCGGA; the primers used for *StSP5G* (transcript ID unknown, ITAG: Sotub05g026730.1.1) were (F) GGTGTGTAGACTTTGGTGTGGTTT, (R) GGCCTCAAGGCACATCCAT and the primers used for *StSP6A* (PGSC0003DMT400060057) were (F) GACGATCTTCGC-AACTTTTACA, (R) CCTCAAGTTAGGGTCGCTTG. Reference genes were *StEIF3e* (PGSC0003DMT400076704, *EUKARYOTIC INITIATION FACTOR 3E*) and *StACTIN* (PGSC0003DMT400010174) and the primers for *StEIF3e* were (F) GGAGCACAG-GAGAAGATGAAGGAG, (R) CGTTGGTGAATGCGGCAGTAGG and the primers for *StACTIN* were (F) GGAAAAGCTTGCCTATGTGG, (R) CTGCTCCTGGCAGTTTCAA. All primers gave a single peak in the melt curve.

Protein detection was done using Western Blot analysis. 100 µl of extraction buffer (20 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 15% glycerol, 0.1 mM EDTA, 29 mM β-mercaptoethanol with 2 mM PMSF and 1% protease inhibitor cocktail (Roche, Basel, Switzerland) was added to approximately 50-100 µg of ground frozen material. After thawing, the samples were vortexed and placed at room temperature for five minutes after which they were centrifuged at 4°C for 10 minutes. The supernatant was collected and loaded onto a 10-wells gel (15 µl sample per well in a 10% SDS-Polyacrylamide gel or 20 µl sample per well in a Mini-PROTEAN, 4-20% TGX gel, Bio-Rad, Hercules, CA). Protein separation was done with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a PowerPac (Basic, Bio-Rad, Hercules, CA) with a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 100 V and was run until the dye ran out of the gel (approximately 110 minutes). The gel was then blotted onto a methanol activated membrane (Immun-Blot PVDF, Bio-Rad, Hercules, CA). The sandwich containing gel, membrane, filter paper and sponges was placed in a transfer buffer (25 mM Tris, 182 mM glycine, 20% SDS, 10% EtOH) and was run for 90 minutes at 300 mA in the PowerPac. After blotting, the membrane was rinsed in demi water and stained with a Ponceau solution (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) to obtain a loading control. After staining, the membrane was rinsed in TBS buffer (TRIS pH 7.5, 150 mM NaCl) and soaked in a milk-TBS mix (5% milk powder ELK Campina in TBS buffer) for one hour.

The membrane was then placed in a falcon tube containing 5 ml 5% milk TBS buffer to which 5 µl of anti-HA antibody was added (Anti-HA-peroxidase, high affinity (3F10), Thermofisher, Waltham, MA) and incubated overnight at 4 ºC. After incubation the membrane was washed three times in TBS-T (30 min total) (TBS with 0,05% v/v Tween-20) and one time for five minutes in TBS. Protein presence was detected using 0.5 ml SuperSignal West Femto Chemiluminiscent substrate (Thermofisher, Waltham, MA) and detected in a chemiluminescence imager (G-box, Syngene, Bangalore, India).

Data analysis

The tuberization and flowering times between treatments were compared with a one-way analysis of variance (ANOVA) and Fisher's protected LSD ($\alpha = 0.05$), for pairwise comparisons. These analyses were computed in GenStat (18^{th} Edition). Due to confined space in the climate chamber, there were no repetitions of light treatments, only biological replicates. Therefore every plant was considered as an independent experimental unit. The qPCR was performed with three biological replicates per time point and treatment, with the exception of 35S::StCOL1-HA samples, which were pooled samples of three plants and had three technical replicates instead. qPCR data were obtained from CFX Manager (Bio-Rad, Hercules, CA) version 3.1 and expression levels were calculated with $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008), where ΔCt is the cycle threshold (Ct) difference between the target genes (*StCOL1, StSP5G, StSP6A*) and the geometric mean of the two reference genes (*EIF3e* and *ACTIN*). For the western blot analysis one sample was used per time point and treatment. The sample was a pool of leaflets from three plants.

Results

Only the night break at the middle of the dark period was able to inhibit tuberization

To determine whether a night break inhibits tuberization by stabilizing StCOL1 in the dark, night breaks given at different moments during the dark period were tested. In the short-day treatment where *S. andigena* was grown under 8 hours

of light and 16 hours of darkness, tuberization occurred after 28 days (Fig. 3.2A). When a night break was applied in the middle of the dark period (NB16), tuberization was inhibited. A night break applied at the beginning of the dark period (NB12) was only able to partially repress tuberization; tuberization was delayed compared to short days and the tubers were small (Fig. 3.2A-B). A night break given at the end of the dark period (NB20) did not inhibit or delay tuberization (Fig. 3.2A). However, tuber biomass was lower than in the short-day treatment (Table 3.1). Under long days no tubers were formed, as was expected

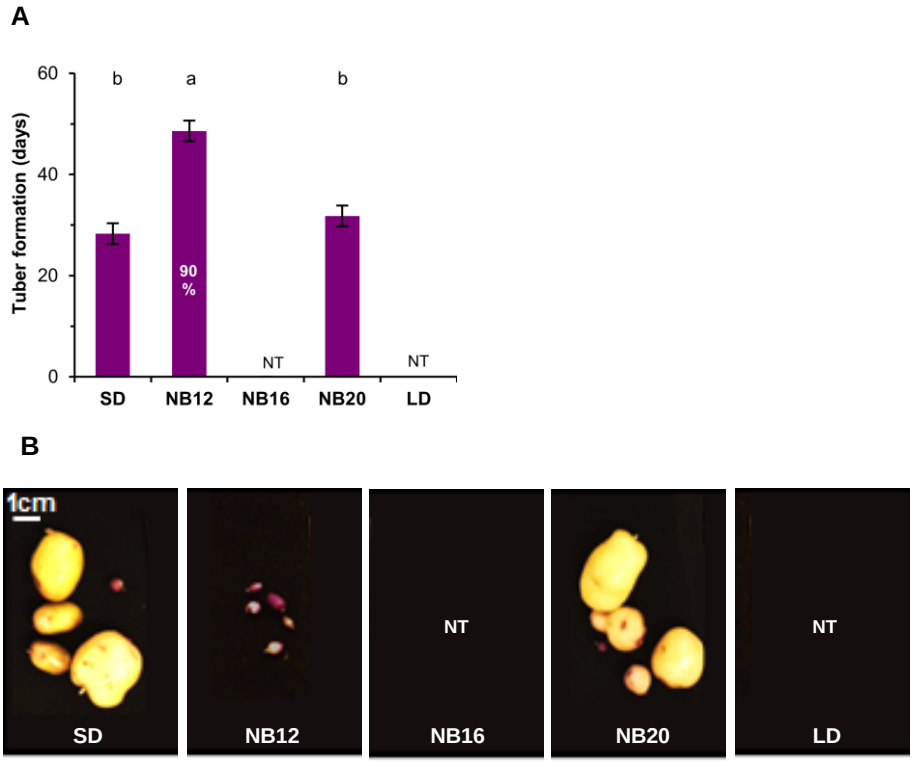


Figure 3.2. Tuberization in light treatments with or without a night break. *S. andigena* plants were grown in short days (SD = 8/16 hours light/dark), in short days with night breaks (30 min) applied at the beginning (NB12), at the middle (NB16) or at the end (NB20) of every dark period, and in long days (LD = 16/8 hours light/dark). The numbers of the night break treatments indicate the hours after the start of the light period. (A) Tuberization time in days after transplanting. NT = no tuberization. Significant differences are indicated with letters ($\alpha = 0.05$, biological replicates, $n = 10$) and error bars indicate the standard error of difference of the ANOVA analysis. The percentage of tuberizing plants is indicated in the bar (no indication means 100% tuberization). (B) Tubers at harvest from one representative plant per light treatment (8 weeks after transplanting). Plants in NB(16) and LD did not tuberize. The light treatments are described in Fig. 3.1.

for *S. andigena*. The time of flower bud appearance was not significantly affected in treatments with a night break compared to the short or long-day treatments (Appendix, Fig. S3.1A). However, the number of leaves formed before the inflorescence did show differences between treatments. The treatments that inhibited tuberization (NB16 and LD) also formed more leaves before the inflorescence than the other treatments (Appendix, Fig. S3.1B). The flower buds were significantly smallest in the short-day treatment (Table 3.1). Plants which received a night break at the beginning of the dark period (NB12) formed the largest flower buds.

Coincidence of the light of a night break and *StCOL1* expression does not always lead to an induction of *StSP5G* and repression of *StSP6A* expression

We hypothesized that a night break given in the middle of the dark period would stabilize StCOL1 and inhibit tuberization, through induction of *StSP5G* and inhibition of *StSP6A*. *StCOL1* expression peaked at the end of the dark period in the short-day treatment (Fig. 3.3A). In the treatment where the night break was given in the middle of the dark period (NB16), *StCOL1* similarly peaked at the end of the dark period (Fig. 3.3G). Although the expression pattern was comparable to the short-day treatment, *StCOL1* was expressed at a low level at ZT(16), the time the night break was applied. In the treatment with the night break at the end of the dark period (NB20), the light of the night break coincided with peak expression of *StCOL1* (Fig. 3.3J), but in the early night-break treatment (NB12), *StCOL1* expression peaked long after the night break was applied (Fig. 3.3D). *StSP5G* was not expressed in the short-day treatment (Fig. 3.3B). This

Table 3.1. Morphological traits of *S. andigena* wild type in light treatments with or without a night break. DW = dry weight. SD = short day, 8/16 hours light/dark. NB = short days with a 30-min night break at ZT(12), ZT(16), or ZT(20) (ZT = Zeitgeber time; hours after start of the light period). LD = long day, 16/8 hours light/dark. Significant differences are indicated with letters ($\alpha = 0.05$, biological replicates, $n = 10$).

	Tubers #		Tuber DW (g)		Shoot DW (g)		Total DW (g)		Flower bud (mm)	
SD	5.6	a	1.79	a	1.9	d	3.7	a	1.3	c
NB12	2.8	b	0.04	c	2.9	a	2.9	b	4.4	a
NB16	0	c	0	c	2.5	b	2.5	c	2.7	b
NB20	5.6	a	1.48	b	2.2	c	3.7	a	2.3	b
LD	0	c	0	c	2.8	a	2.8	b	3.1	b

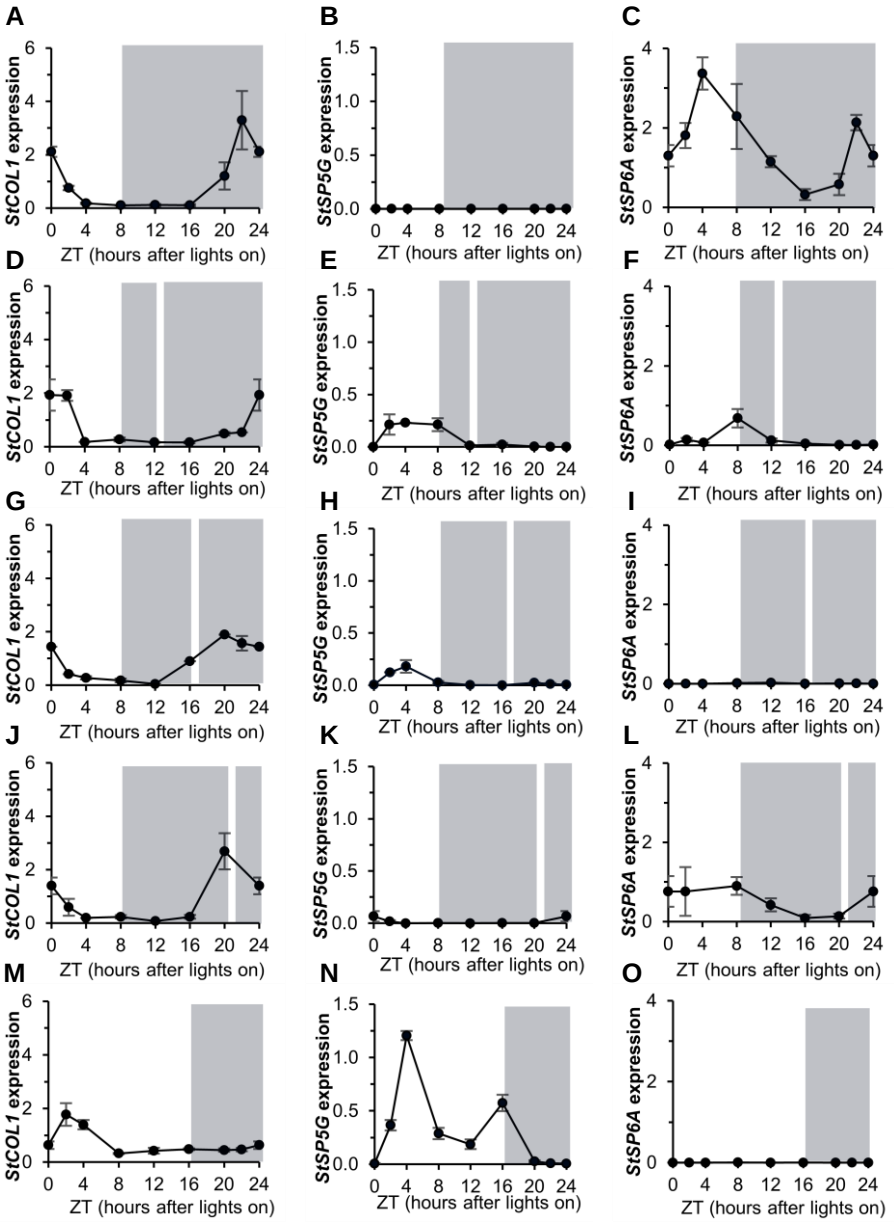


Figure 3.3. Gene expression in light treatments with or without a night break. *StCOL1, StSP5G* and *StSP6A* expression four weeks after transplanting in different light treatments in *S. andigena* wild-type plants. Treatments are a SD: a short day with 8/16 hours light/dark (A-C), NB(12): a short day with a night break at ZT(12) (ZT = Zeitgeber time, hours after lights on) (D-F), NB(16): a short day with a night break at ZT (16) (G-I), NB(20) a short day with a night break at ZT(20) (J-L) and a LD: a long day with 16/8 hours light/dark (M-O). The night breaks were applied every night throughout the duration of the experiment. Error bars indicate standard errors of the mean (biological replicates per time point, $n = 3$).

corresponded to an elevated *StSP6A* expression (Fig. 3.3C) and the finding that these plants tuberized. In the treatment with the night break in the middle of the dark period (NB16), *StSP5G* was upregulated in the morning, and *StSP6A* was not expressed, which corresponded to the inhibition of tuberization (Fig. 3.3H-I). In the late night-break treatment (NB20), *StSP5G* was hardly upregulated (Fig. 3.3K), even though the peak *StCOL1* expression coincided with the light of the night break. *StSP6A* in this treatment was expressed (Fig. 3.3L), albeit lower than in the short-day control. *StCOL1* expression in the early night-break treatment (NB12) did not coincide with the light of the night break, but *StCOL1* expression in the early night-break treatment was induced in the light period at ZT(2), which was not the case in the other treatments, except the long day. *StSP5G* was induced in the early night-break treatment (Fig. 3.3E). However, even though *StSP5G* was expressed higher than the regular night-break treatment, *StSP6A* was still partially induced (Fig. 3.3F). The long-day *StCOL1* expression peaked at ZT(2), and *StSP5G* was highly expressed compared to the other treatments (Fig. 3.3M-N). As expected, *StSP6A* was not expressed in long days, corresponding to the inhibition of tuberization (Fig. 3.3O). Although both the long-day treatment and the night-break treatment in the middle of the dark period (NB16) did not tuberize, *StSP5G* expression in the night-break treatment was very low compared to the long-day treatment. The expression results show that coincidence of light and *StCOL1* expression does not always lead to an induction of *StSP5G* and strong repression of *StSP6A* and therefore also not to inhibition of tuberization.

StCOL1 protein is stabilized during a night break

We expected a night break to inhibit tuberization by stabilization of StCOL1 protein in the dark period. We determined the presence of StCOL1 protein using Western Blotting in a line overexpressing StCOL1 (35S::StCOL1-HA). By using an overexpressing line we were able to observe when the StCOL1 protein was absent and thus determine when it was degraded. By comparing this information with the *StCOL1* expression pattern in wild-type lines, it could be determined when StCOL1 protein could be present in the leaves. Under short-day conditions in the overexpressing line, StCOL1 was present in the light and degraded in the dark period (Fig. 3.4). In all night-break treatments, StCOL1 was stable during the night break, but was readily degraded as soon as the night break came to an

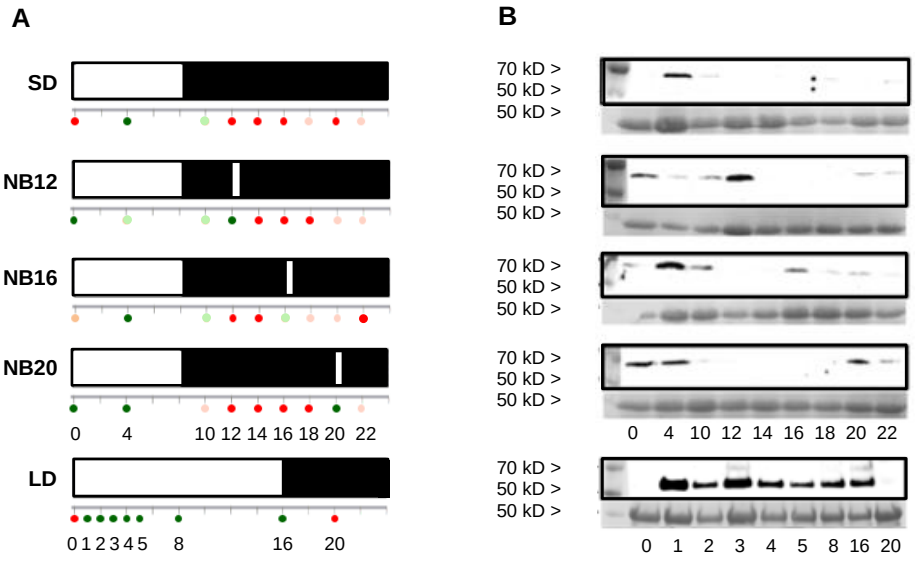


Figure 3.4. StCOL1 protein in light treatments with or without a night break. Western blot detection of StCOL1 protein in 35S::StCOL1-HA plants in an *S. andigena* background, four weeks after transplanting. (A) A schematic representation of protein presence is given at several ZTs (ZT = Zeitgeber time, hours after start of the light period) (red: no StCOL1, pink: little StCOL1, light green: some StCOL1, green: clear StCOL1 presence). SD = short day, NB = night break, LD = long day. The night breaks were applied every night throughout the duration of the experiment. Numbers behind NB indicate the ZT at which the 30-minute night break is given (B) Western blot. Protein presence at several ZTs based on a pooled sample of three biological replicates. The sampled time points in the long day differ from the short-day treatments. A ponceau stain is given as a loading control (sub-unit Rubisco ~56 kDa (reviewed in: Spreitzer, 1993)).

end. StCOL1 seemed to be present in very low levels at the end of the dark period in all treatments. In almost all short-day treatments, StCOL1 was still vaguely present at the beginning of the dark period (ZT(10)). This was clearest in the treatments with the least tuberization (NB16 and NB12).

StSP5G in plants overexpressing *StCOL1* is similar under long and short days, but *StSP6A* is upregulated in short days

The *StCOL1* overexpressing line showed a high expression of *StCOL1* throughout the day in both long and short days (Fig. 3.5A). In both long and short days *StSP5G* was induced as well (Fig. 3.5B). However, *StSP6A* expression was only repressed in the long-day plants (Fig. 3.5C). In short-day plants, *StSP6A* was upregulated and tubers were formed (Fig. 3.5C-E). However, upregulation was lower and

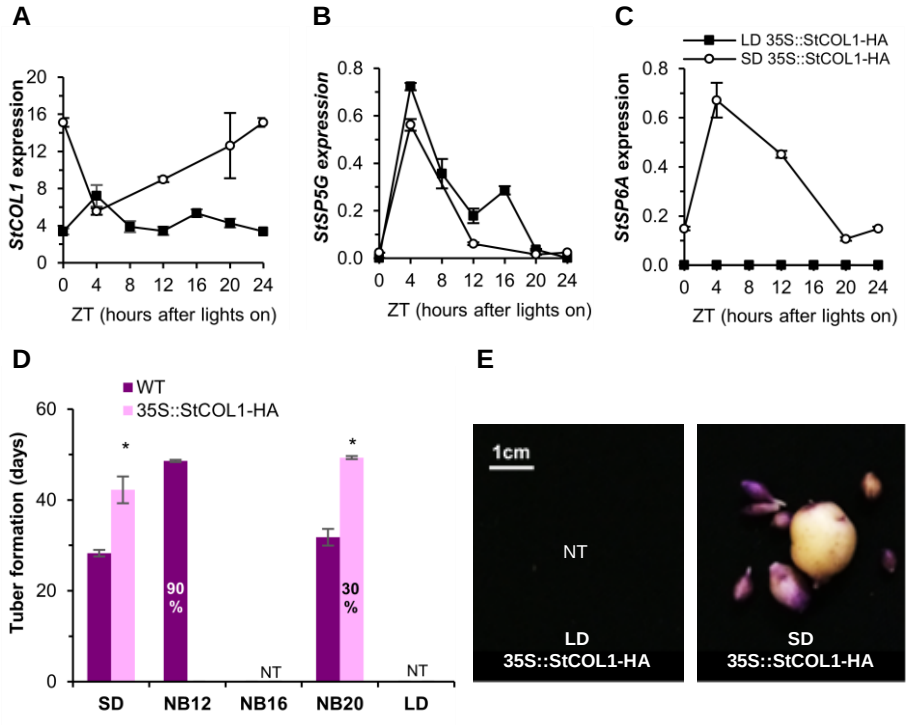


Figure 3.5. Gene expression and tuberization in *StCOL1* overexpressing plants. Gene expression four weeks after transplanting and tuberization in 35S::StCOL1-HA plants in an *S. andigena* background in different light treatments. *StCOL1* (A), *StSP5G* (B) and *StSP6A* (C) expression in short days (SD, open circle) and long days (LD, closed square). *n* = 3, one pool of three biological replicates with three technical replicates was analyzed per time point. (D) Tuberization in SD, in SD with a night break (NB12, NB16 and NB20) and in LD (LD), in wild-type (WT) and 35S::StCOL1-HA *S. andigena*. The number behind the NB indicates at what ZT (ZT = Zeitgeber time, hours after start of the light period) the 30-minute night break was applied. NT = no tuberization. The asterisk indicates a significant difference between the wild type and the overexpressing line (α = 0.05, biological replicates, *n* = 10). If not all plants tuberized, the percentage that did is indicated. Error bars indicate standard error of the mean. (E) Tubers of a 35S::StCOL1-HA plant in long and short days.

tuberization was delayed compared to wild-type plants (Fig. 3.3C and 3.5C-D). Tuberization in the *StCOL1* overexpressing plants was also delayed in the night-break treatments. Tuberization in the late night-break treatment (NB20) occurred more than two weeks later than in the wild-type plants, while tuberization in the early night-break treatment (NB12) was totally inhibited when *StCOL1* was overexpressed (Fig. 3.5D).

Discussion

Inhibition of tuberization by a night break cannot be explained by coincidence between *StCOL1* expression and light

A night break applied in the middle of the dark period in short days inhibits tuberization (Batutis and Ewing, 1982). Our results show that a night break given in the middle of the dark period suppresses expression of the gene encoding the tuberization signal StSP6A and inhibits tuberization (Fig. 3.2A and 3.3C). We hypothesized that the functioning of a night break on tuberization could be explained by the coincidence of the light given during the night break, and the high *StCOL1* expression in the dark period. The light of the night break would stabilize StCOL1 protein, which in turn would lead to induction of *StSP5G,* and the inhibition of *StSP6A* and tuberization. Our results show that *StCOL1* was expressed at a low level during the application of a classic night break in the middle of the dark period (NB16) (Fig. 3.3G). Furthermore, the StCOL1 protein was stable at the time of night-break application, indicating StCOL1 protein could induce *StSP5G* expression and repress *StSP6A*, which matches our expression results. However, the short day without a night break had a similar low level of *StCOL1* expression in the beginning of the day but did have upregulated *StSP6A* and tuberization (Fig. 3.3A). As suggested by the coincidence model, peak *StCOL1* expression has to coincide with light to induce *StSP5G* and inhibit tuberization (Imaizumi and Kay, 2006, Abelenda *et al.,* 2016), but in both the short day and in NB16 treatment, peak *StCOL1* expression took place in the dark. Thus, coincidence of peak *StCOL1* expression and light cannot explain the functioning of a night break.

An alternative explanation is that light activates PHYB, which is only slowly deactivated by dark reversion (Ruddat *et al.,* 1997) and can remain active to stabilize StCOL1 during peak *StCOL1* expression later in the dark period. PHYB was shown to be involved in StCOL1 stabilization in potato, and in rice mediated night-break inhibition of flowering (Ishikawa *et al.,* 2005, Abelenda *et al.,* 2016). Furthermore, a recent study has shown that night break delayed flowering in tomato is regulated through PHYB (Cao *et al.,* 2018). In the case of a prolonged effect of PHYB mediated StCOL1 stabilization, not the coincidence of light and peak *StCOL1* expression, but applying light just before *StCOL1* peak expression is crucial. In the short-day treatment light is only applied after the *StCOL1* peak,

while in the NB16 treatment light is applied before the *StCOL1* peak. If this hypothesis is correct we would expect StCOL1 to be present in the period between night-break application (ZT(16)) and peak *StCOL1* expression (ZT(20)) (Fig. 3.3G). However, western blot analysis showed that StCOL1 degradation already took place at ZT(18) (Fig. 3.4), making it unlikely that enough StCOL1 would be stabilized during peak *StCOL1* expression to inhibit tuberization. These findings are supported by the unexpected results of the late night-break treatment NB20, where coincidence of light and peak *StCOL1* expression at ZT(20) did not lead to high *StSP5G* expression or inhibited tuberization, even though StCOL1 protein was present during the night break at ZT(20) (Fig. 3.4). Plants in the NB20 treatment tuberized just as fast as in the short-day treatment. These findings do not support the theory that coincidence of light and peak *StCOL1* expression or even application of light just before *StCOL1* expression leads to inhibition of tuberization. Instead, these results imply that other factors than StCOL1, StSP5G and StSP6A are involved in night-break inhibited tuberization. Expression data from the early night-break treatment NB12 may support this idea. The night break did not coincide with *StCOL1* expression (Fig. 3.3D), but *StSP5G* was upregulated and *StSP6A* expression was repressed compared to the short day without a night break (Fig. 3.3E-F). Furthermore, tuberization was delayed (Fig. 3.2A). Although peak *StCOL1* expression took place right at the end of the dark period, or the beginning of the light period, it is not sure that in this case light coincides with peak expression. It could be the case that the night break at the beginning of the night caused the repression of tuberization instead. In tomato it was shown that night breaks delayed flowering through PHYB mediated upregulation of *SP5G* (Cao *et al.,* 2018), which is the tomato homolog of *StSP5G* (Abelenda *et al.,* 2016; Soyk *et al.*, 2017). It could be the case that *StSP5G* is also induced by PHYB in an early night break, and that this induction happens independently of StCO. However, it is surprising that *StSP6A* expression and tuberization are only partially repressed in the early night-break treatment, considering *StSP5G* expression is higher than in the NB16 treatment, where *StSP6A* is fully repressed and tuberization is fully inhibited (Fig. 3.2A and 3.3E-F, H-I).

Light stabilized StCOL1 in the night, cannot explain the inhibitory function of a night break. The lack of *StSP5G* upregulation despite the coincidence of *StCOL1* and light and the presence of StCOL1 protein at ZT(20) in the NB20 treatment indicates that there is an extra level of control between StCOL1 and

StSP5G that still needs elucidating or that *StSP5G* is regulated by a transcription factor other than StCOL1. Furthermore, the upregulation of *StSP6A* despite upregulated *StSP5G* in the NB12 treatment suggests that *StSP5G* expression alone does not determine *StSP6A* induction.

StCOL1 overexpressing plants induce *StSP5G* in both long and short days, but *StSP6A* and tuberization are only induced in short days

In the night-break treatments, the coincidence of *StCOL1* and light did not always lead to strong repression of *StSP6A* expression and inhibition of tuberization. This lack of correlation was also seen in the plants overexpressing *StCOL1*. Although *StCOL1* was expressed throughout the day in both short- and long-day conditions, and *StSP5G* was expressed in both day lengths, *StSP6A* was only induced in short days. This confirms our earlier finding that upregulation of *StSP5G* does not necessarily lead to repression of *StSP6A.* StSP5G is not a transcription factor and needs an additional factor to affect *StSP6A* expression. Abelenda *et al.* (2016) suggested that StSP5G represses an inducer of *StSP6A*. An additional control on post-translational level may determine whether StSP5G is able to repress *StSP6A*. Alternatively, *StSP6A* may not be induced at all in long days, in this case StSP5G may partially repress *StSP6A* in short days, while *StSP6A* is not expressed at all in long days. However, *StCOL1 RNAi* lines with very low *StSP5G* expression had upregulated *StSP6A* in long days (Abelenda *et al.,* 2016), suggesting that *StSP6A* expression is not only activated in short days.

Another possibility explaining *StSP6A* expression in *StCOL1* over-expressing lines in short days is that another factor that is not repressed by StSP5G, induces *StSP6A* in short days. The BEL1-like transcription factor, StBEL5, is involved in tuberization control in potato (Chen *et al.,* 2003). StBEL5 had been reported to induce *StSP6A* in the leaves and in the right conditions the RNA transcript is transported to the stolons and thought to induce *StSP6A* there (Hannapel *et al.,* 2017). It was found that StBEL5 transcript is stabilized by a polypyrimidine tract-binding protein (StPTB), which accumulates under short-day conditions (Banerjee *et al.*, 2006, Cho *et al.,* 2015) and therefore in short days, StBEL5 transcript is increased (Chen *et al.,* 2003). As *StSP6A* expression happens downstream of *StCOL1*, induction of *StSP6A* by StBEL5 in short days may permit some *StSP6A* to be expressed, even when *StCOL1* is overexpressed and *StSP5G* is upregulated. Kloosterman *et al.* (2013) propose that CYCLING DOF

FACTOR 1 (StCDF1), which represses *StCOL1* in short days, may also act directly on *StSP6A* expression. Subsequently, the tuber inducing role of StCDF1 by repressing *StCOL1* may be compensated by the overexpression of *StCOL1,* but StCDF1 may also directly activate *StSP6A* in short days. However, if *StSP6A* is upregulated by StCDF1 in short days, it is not clear how a short day with a night break can inhibit tuberization, as the night break did not affect *StCOL1* expression and thus probably also not upstream *StCDF1* expression.

Our results indicate additional regulation exists between StSP5G and *StSP6A* expression, which is also under photoperiodic control.

A night break applied at the middle of the dark period has the strongest inhibitory effect on tuberization

Before anything was known about the molecular control behind tuberization, a night break was claimed to inhibit tuberization by dividing a long night into two short nights (Jackson, 1999). Our results show that the night break was only able to fully inhibit tuberization when given at the middle of the night period (NB16). Similar findings were made in the short-day plants rice and chrysanthemum, where a night break given at the middle of the night period had the strongest inhibitory effect on the flowering (Horridge and Cockshull, 1989, Ishikawa *et al.,* 2005). In long-day plant wheat, several night breaks were tested and the most successful night break was the one given at the middle of the night period (Pearce *et al.,* 2017). The action of a night break may not be regulated through coincidence between *StCOL1* and light, but through a mechanism involving the night length. However, in our experiments a different tuberization response was seen in the NB20 and NB12 treatments, which both had the same length of dark periods. The most successful of the two in repressing tuberization (NB12), first had a short dark period followed by a night break and a longer dark period. Instead of the length of the dark periods, the time until the first light period may be important for successful inhibition of tuberization.

An unknown factor controlled by the duration of the dark period may affect tuberization

Pearce *et al.* (2017) found the length of darkness before the night break increased the expression of *PPD-B1,* an allelic variant of *PHOTOPERIOD1 (PPD1)*

whose expression leads to *FLOWERING LOCUS T1* (*FT1)* induction in wheat and may be comparable to *StCOL1* in potato. However, the increase in *PPD-B1* did not correlate to flowering time, suggesting that other circadian clock genes also play a role in night-break induced flowering in wheat. Interestingly, more parallels exist between night breaks in wheat and potato. Although *StCOL1* was expressed at a low level during a classic night break, *StSP5G* was induced and tuberization was inhibited. In wheat, the greatest night-break mediated flower induction was observed in the middle of the night when *PPD1* expression was low (Pearce *et al.,* 2017). The authors suggested the effect of the night break was gated by one or more unknown circadian clock genes, which may be the case for potato as well.

Recent studies in short-day Chrysanthemum suggest that flowering depends on the duration of the night. Flowering was only possible when the night length exceeded a photosensitive phase for induction of anti-florigen (Higuchi *et al.,* 2013). The gate for maximal induction of anti-florigen opens at a constant time after dusk, regardless of the period of light preceding it. Coincidence with light during this period, either when nights are short or when a night break is applied, leads to induction of anti-florigen. A similar mechanism could take place in potato. An unknown factor may be expressed as soon as the dark period starts, with peak expression taking place in the middle of a long night (ZT16). Coincidence with the light of a night break may lead to the induction of an anti-tuberigen in potato, which in this case may be *StSP5G*. In long days, peak expression of this unknown factor coincides with the light of dawn, also leading to *StSP5G* induction. An early night break (NB12) may coincide with an early low expression of this unknown gene, while during the late night break (NB20) the photosensitive period is over, explaining the low *StSP5G* expression, even while StCOL1 is stabilized.

Although a mechanism involving an additional factor influenced by the night duration is plausible, not all results can be explained. For instance, why do *StCOL1* overexpressing plants have similar high *StSP5G* levels if only in long days the additional factor induces *StSP5G*? Possibly both this factor and StCOL1 act on *StSP5G*. Alternatively, this additional factor may stabilize or activate StSP5G or even repress *StSP6A* independently of StSP5G, explaining why *StSP6A* is fully repressed in long days in *StCOL1* overexpressing lines, while in short days some *StSP6A* expression can still occur. A complex mechanism may take place where multiple factors act on *StSP5G* and *StSP6A*, complicating the existing StCOL1 controlled model explaining potato tuberization. An additional factor that is

controlled by the duration of the dark period may be a missing link in the control of photoperiodic tuberization.

Conclusion

Our results show that coincidence between *StCOL1* expression and light cannot explain why a night break inhibits tuberization in short days. Night-break treatments that were given during peak expression of *StCOL1* did not strongly induce *StSP5G* nor substantially repress *StSP6A* expression. Furthermore, plants overexpressing *StCOL1* still expressed *StSP6A* in short days and were able to tuberize, even though *StSP5G* expression was almost identical to expression in long-day plants, which did not tuberize. Our findings suggest there is an additional level of control between *StCOL1*, *StSP5G* and *StSP6A* expression, which determines whether or not a plant tuberizes.

Acknowledgements

This research is part of the FlowerPot project which was supported by the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO), Signify, Solynta, Beekenkamp Plants and Incotec. Thanks to Signify for providing the LEDs for this project. Special thanks to José Anton Abelenda for use of the transgenic lines and critical advice about the light treatments and to Francisca Reyes Marquez for advice and help with the Western Blot analysis.

Appendix

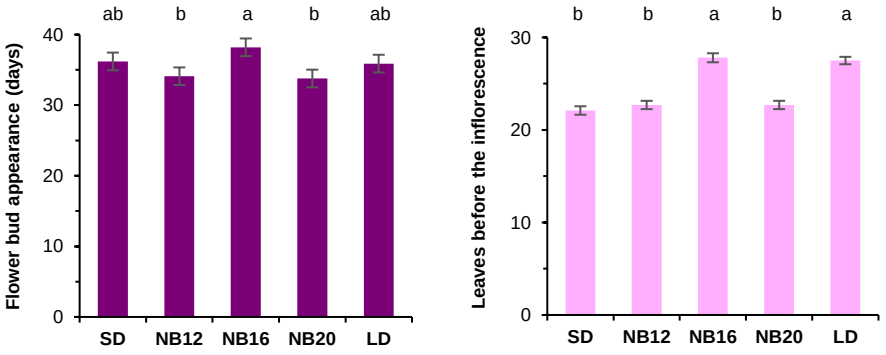


Figure S3.1. Flowering time in *S. andigena* wild-type plants grown in light treatments with or without a night break. Plants were grown in short days (SD = 8/16 hours light/dark), in short days with night breaks (30 min) applied in the beginning (NB12), in the middle (NB16) or at the end (NB20) of the dark period, and in long days (LD = 16/8 hours light/dark). The night breaks were applied every night for the duration of the experiment. The numbers of the night-break treatments indicate how many hours after the start of the light period the light treatment was given. (A) Flower bud appearance time in days between transplanting and appearance of the first flower bud. (B) Flowering in the number of leaves formed before the inflorescence. Significant differences are indicated with letters ($\alpha = 0.05$, biological replicates, $n = 10$).

4. High light accelerates potato flowering independently of the *FT*-like flowering signal *StSP3D*

Faline Plantenga, Sara Bergonzi,
Christian Bachem, Richard Visser,
Ep Heuvelink and Leo Marcelis

A revised version was accepted for publication in Environmental and Experimental Botany, 2019

Abstract

Little is known on the environmental control of potato flowering. With the recent development of hybrid breeding in potato, more knowledge on potato flowering is desired. This research aims to elucidate the effect of the daily light integral (DLI) on potato flower initiation time and determine which mechanisms underlie this control. In this study we hypothesized that a high DLI accelerates flower initiation by increasing carbohydrate levels in the plant. Furthermore, we hypothesized that an increase in carbohydrates upregulates the proposed flowering time gene *StSP3D*, which in turn may induce flowering. We grew potato plants in climate chambers to compare flower initiation in different DLIs under short and long days. We measured the time until the first appearance of the flower buds and the number of leaves formed before the inflorescence. Additionally, we determined sucrose and starch concentrations and measured gene expression changes of *StSP3D* and of *StTPS1*, a gene involved in sugar mediated flowering control. Potato plants silenced in *StSP3D* were used to determine whether DLI-mediated flowering time was regulated through *StSP3D.* Increasing the DLI clearly accelerated flowering in potato, while the role of carbohydrates (sucrose and starch) and *StTPS1* in this control was inconclusive. Although *StSP3D* was upregulated under high DLIs, transgenic lines silenced in *StSP3D* also showed accelerated flowering under high DLIs. We therefore concluded that a high DLI accelerates potato flowering and this acceleration happens independently of *StSP3D* upregulation.

Keywords: potato flowering, daily light integral (DLI), StSP3D, StTPS1, sucrose

Introduction

Potato plants can reproduce asexually through tuberization, and sexually through flowering. Tubers are used for consumption, processing and as starting material for vegetative propagation. As a result, mechanisms of the regulation of tuberization have gained more attention than those of potato flowering. However, with recent developments in hybrid breeding of potato, where seeds are the starting material, more research is needed on the regulation of potato flowering (Lindhout *et al.*, 2011).

Although little is known about the transition to sexual reproduction in potato, light is expected to be an important factor. The amount of light has been found to affect flowering time in several species (Bernier *et al.*, 1993; Levy and Dean, 1998; Adams *et al.*, 1999) and indeed, the amount of light has also been found to affect potato flowering. In several experiments, reducing the amount of light that was available to potato plants reduced the development of flower buds (Demagante and Van der Zaag, 1988; Turner and Ewing, 1988). However, the effect on flower initiation time was not considered. Here, we investigate how the daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$) affects potato flower initiation and elucidate how this control takes place.

Although plants possess photoreceptors that are able to sense several aspects of light and relay signals to the plant (Atwell *et al.*, 1999), little is known on how differences in the amount of light can control plant signaling. A possible method could be through the formation of carbohydrates (Thomas, 2006). When plants receive more light throughout the day, more light can be used for photosynthesis and the synthesis of carbohydrates like sucrose, glucose, fructose and starch. Although necessary for plant growth in general, carbohydrates like sucrose can also act as signaling molecules and have been found to alter gene expression and enzymatic activity (Smeekens, 2000). In fact, sugars have been shown to be involved in flowering control in several plant species (Bernier and Périlleux, 2005; Chincinska *et al.*, 2008; Srikanth and Schmid, 2011; Bouché *et al.*, 2016). High light treatments increased sucrose accumulation in the apical meristem of *Sinapis alba* (Havelange and Bernier, 1983). Other studies showed that supplying exogenous sucrose to plants, allowed them to flower under suboptimal light conditions (King and Bagnall, 1996; Thomas, 2006). Considering these studies, it is likely that if the DLI affects flowering in potato, this control is in part regulated by carbohydrates.

In potato little is known about carbohydrate regulated flowering. Sucrose transporters play a role in potato flowering, considering potatoes silenced in the sucrose transporter StSUT4 flowered early (Chincinska *et al.*, 2008). These *StSUT4 RNAi* plants had a strongly increased sucrose efflux from the leaves at the end of the light period, leading to an increase in sucrose levels in sink organs. Furthermore the sucrose levels in the apical meristem increased just before the onset of flowering and peaked earlier in *StSUT4 RNAi* lines (Chincinska *et al.*, 2008). How these changes in the plant's sucrose status eventually lead to a change in potato flowering has yet to be discovered.

In *Arabidopsis thaliana* the carbohydrate pathway and its control on flowering are understood in more detail (Bouché *et al.*, 2016). A proposed mechanism for sugar mediated flower induction involves the signaling metabolite trehalose-6-phosphate (T6P). T6P levels are closely correlated with sucrose levels in vegetative plant tissues and the shoot apical meristem, and therefore T6P may act as a proxy for sucrose status in the plant (Lunn *et al.*, 2006; Matsoukas *et al.*, 2012; Bolouri Moghaddam and Van den Ende, 2013). The first step in T6P biosynthesis is catalyzed by TREHALOSE-6-PHOSPHATE SYNTHASE 1 (TPS1) and expression of *TPS1* is essential for transition to flowering (Dijken *et al.*, 2004; Wahl *et al.*, 2013). It is not yet fully understood where *TPS1* acts in the flowering pathway, but T6P is required in the leaves for induction of the flowering time gene *FLOWERING LOCUS T (FT)* (Wahl *et al.*, 2013; Yu *et al.,* 2015). In potato an *FT-like* homolog called *SELF-PRUNING 3D* (*StSP3D),* has been identified and was suggested to be the flower inducing signal (Navarro *et al.,* 2011). Although the expression of this gene is under photoperiodic control in the leaves, the authors propose *StSP3D* may be more strongly regulated by other environmental cues and mention irradiance as a possible candidate. Consequently, flowering time in potato may be controlled by light amount and this control may be regulated through sucrose and *StTPS1,* and ultimately through upregulated expression of the flowering time gene *StSP3D*.

The effect of the DLI on potato flowering is not well described and it is not known which mechanisms underlie this control. In this research we tested the hypothesis that a higher DLI, in short and long days, accelerates flower bud appearance in potato by increasing carbohydrates in the plant and inducing the flowering time gene *StSP3D*. We recorded the flower bud appearance time and the number of leaves formed before the inflorescence, under different light conditions. Furthermore, we determined whether the high light conditions

increased the carbohydrate status in the plants by measuring sucrose and starch concentration in the leaves and shoot apex, and by measuring *StTPS1* expression in the leaves. *StSP3D* expression was also measured in leaf tissue to investigate a correlation between its transcriptional level and flowering. Finally, potato plants silenced in *StSP3D* were grown in low- and high-light conditions to determine whether the DLI affected flowering time through *StSP3D.*

Materials and methods

Plant materials, plant transformations and growth conditions

A tetraploid obligate short-day tuberizing *Solanum tuberosum* L. ssp. *andigena* (*S. andigena*) and a diploid *S. tuberosum* ssp. *tuberosum* clone CE3027 were used. CE3027 is part of a mapping population which was formed by crossing diploid parent genotypes C (USW5337.3) and E (77.2102.37) (Celis-Gamboa, 2002). Unlike *S. andigena,* CE3027 has abundant flowering and is diploid, making it more comparable to genotypes used for hybrid breeding in potato. Next to the two wild-type genotypes, two transgenic lines in an *S. andigena* background were used that were silenced in the flowering signal *StSP3D* (*StSP3D RNAi* #5 and *StSP3D RNAi* #7).

To generate the transgenic lines, a fragment of 311 bp amplifying the last exon and 3' UTR of the *StSP3D* gene of *S. andigena* was amplified by PCR and cloned into the donor vector pENTR221 to generate an entry clone (Gateway® Thermo Fisher Technology). The primers (F) GGGGACAAGTTTGTACAAAAAAGC-AGGCTCACCGTTCAGACAATTAGGTCGACA and (R) GGGGACCACTTTGTACAAGA-AAGCTGGGTAAGTAGTAGAGATTGGTGGTT, including the sequences necessary for the BP-cloning, were used. To generate the RNAi expression clone, LR-reaction was performed using the destination vector pK7WIWG2. *Agrobacterium tumefaciens* strain AGL0 was transformed with the obtained expression vector and internode explants from *S. andigena* transfected as previously described to generate transgenic potato plants (Visser, 1991).

Plant material was propagated *in vitro* in plastic containers containing MS20 agar medium (Murashige and Skoog, 1962). Plantlets were maintained in a climate chamber at 24°C in long days (16 h light 8 h dark) under fluorescent tubes with a photosynthetic photon flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After

approximately two weeks of growing, plantlets were transplanted to small square pots (7 x 7 x 8 cm) in *Experiment 1* and to larger round pots (17cm Ø x 12.5 cm) pots in *Experiment 2* (see next section). The pots were filled with a clay-peat mixture (pH is 5.7). After transplanting, pots were placed in a climate chamber and were watered manually. After two weeks on soil, liquid fertilizer was applied once a week (Hydro Substrafeed™: 35.1% N, 40.4% S, 42.5% P, 35.8% K, 17.5% CaO with an EC (electrical conductivity) of 2.0 $dS \cdot m^{-1}$). The air temperature was 20ºC day and night in Exp. 1 and 22ºC day and 18ºC night in Exp. 2. The relative air humidity was 70%. Light was provided by white and red LEDs (light emitting diodes) (Philips GreenPower LED production module 120 cm DeepRedWhite-2012, the white LEDs were phosphor-coated blue LEDs). Light intensity was measured at plant canopy height with a quantum sensor (LI-COR Biosciences, LI-190SB Quantum, LI-1400 Datalogger). The height of the LED modules was adjusted every two weeks to maintain the required light intensity. Pots were rotated three times a week to ensure uniform light distribution. Side shoots were removed from the plants as soon as they appeared.

Experimental set-up

Two experiments were performed. The first was performed to determine the influence of light treatments on flowering time and carbohydrate concentrations in the plant. The second was done to confirm these findings in different genotypes and determine whether flowering time in plants silenced in *StSP3D* was affected by the light treatments.

Experiment 1

CE3027 wild-type plants were grown in a climate chamber with long days (16 hours light) at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After one week the plants were distributed over five light-tight compartments (1.3 m^2 per compartment) within the climate chamber. Each compartment had a different light treatment which consisted of combinations of day length, light intensity and DLI (light intensity x day length) (Table 4.1A): three short-day treatments (8 h light) with light intensities of 100, 200 or 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a short day with 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 15-minute night break of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle of the dark period (sensed as a long day for tuberization), and a long-day treatment (16 h light) with 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Table 4.1. Light conditions and genotypes. (A) Experiment 1 and (B) experiment 2. Bars indicate day length, where white is the light period and grey is the dark period. SD = short day 8/16 hours light/dark, SD + NB = short days with a night break of 15 minutes light in the middle of the dark period, LD = long day, 16/8 hours light/dark. WT = wild type. The SD + NB treatment mimics a LD treatment. DLI = daily light integral (light intensity x photoperiod). The transgenic lines in (B) are in an *S. andigena* background.

A

Code	Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	DLI ($mol \cdot m^{-2} \cdot d^{-1}$)	Day length	Genotypes
SD 2.9	100	2.9	SD	WT CE3027
SD 5.8	200	5.8	SD	
SD 11.5	400	11.5	SD	
SD+NB 5.8	200	5.8	LD	
LD 11.5	200	11.5	LD	

B

Code	Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	DLI ($mol \cdot m^{-2} \cdot d^{-1}$)	Day length	Genotypes
SD 5.8	200	5.8	SD	WT *S. andigena*
SD 11.5	400	11.5	SD	WT CE3027
LD 11.5	400	11.5	LD	*StSP3D RNAi* #5
LD 23	400	23	LD	*StSP3D RNAi* #7

Experiment 2

Four light conditions were tested (2 m^2 per compartment). WT *S. andigena,* WT CE3027, *S. andigena StSP3D RNAi* #5 and *S. andigena StSP3D RNAi #7* were grown in each light treatment, which consisted of two short days with a light intensity of 200 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light and two long days with a light intensity of 200 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light (Table 4.1B). Exp. 2 was repeated in an identical climate chamber with the wild-type plants, two weeks after the start of the first experimental round. The environmental conditions were identical in both experimental rounds.

Some plant measurements from Exp. 1 and 2 were used in Plantenga *et al.* (in prep, 2018). However, flower bud development and anthesis (open flowering stage) were described in that paper, while the current paper focusses on an earlier stage of flowering, the flower initiation time.

Daily measurements and sampling

Experiment 1

Six plants were destructively sampled for carbohydrate analysis after two, four and six weeks (18 plants in total). After sampling for the shoot apex, the plant could not be used for further measurements and had to be discarded. Eleven

plants remained for non-destructive phenotyping and the destructive harvest after eight weeks of growing. Six out of eleven plants were sampled for carbohydrate analysis at the final harvest. For the carbohydrate sampling three pools of two plants were made per treatment. Samples were collected at the end of the light period. The bottom left leaflet on the sixth leaf from the top was collected for the leaf sample and the shoot apex was collected by cutting of the tip of the plant and removing as much leaf material as possible. The leaf and shoot apex material was frozen in liquid nitrogen and stored at -80ºC. Two weeks after transplanting, plants were re-potted to 17cm Ø pots to ensure proper growth. Plants used for phenotyping were examined three times a week for flower bud appearance. Also the number of leaves formed below the flower buds was noted. At the destructive harvest the dry weight of stems, leaves and tubers was determined.

Experiment 2
Six plants (three pools of two) were sampled per treatment in the wild-type lines *S. andigena* and CE3027. As opposed to Exp. 1, only the leaves and not the shoot apices were sampled. Therefore plants could be retained after sampling and used to determine the flowering phenotype. Sampling was done three, five and seven weeks after starting the experiment. Next to sampling for sugar analysis, leaf samples were taken for gene expression analysis. This was done at 1h after the lights turned on (which corresponds to a peak in the *StSP3D* expression, Appendix, Fig. S4.1), after three and five weeks after starting the experiment. The 5th leaf from the top was sampled for gene expression and one pool was made of leaves from three plants. After sampling, leaf material was frozen in liquid nitrogen and stored at -80ºC. Measurements of flowering time and number of leaves before flowering as well as the final harvest were performed as in Exp. 1.

Carbohydrate analysis

Carbohydrate concentrations were measured in the leaves and in the shoot apex. After storage at -80ºC, plant material was freeze dried (freeze dryer, Modulyo, Edwards) and powdered using a ball mill. Approximately 15 mg of ground leaf material was mixed with 5ml 80% EtOH (ethanol) in a shaking water bath at 80ºC for sugar extraction. For apex material approximately 3 mg of ground material was mixed with 1 ml 80% ethanol. After centrifugation, the supernatant

containing the soluble sugars was vacuum dried (SpeedVac concentrator SPD 2010, Thermo Scientific, ThermoFisher). The remaining pellet was dissolved in 1ml Milli-Q water and diluted 0-10 times in Milli-Q water, depending on the initial sample size. Sucrose, fructose and glucose were quantified in a high performance ion chromatograph (HPIC, ICS-5000, Thermo Scientific, Thermofisher). This was done in an anion exchange column at 25ºC (250x2mm column, CarboPac PA1, Thermo Scientific) with 100 mM NaOH at a flow rate of 0.25 ml·min^{-1}. Detection was carried out by pulsed amperometry. Chromeleon 7.1 Software (Thermo Scientific) was used to analyze the chromatograms and quantify the amount of soluble sugars in the samples. The pellet that remained after dissolving the soluble sugars for the first time in ethanol was used for starch determination. After the supernatant was used for sugar detection, the remaining supernatant was discarded and the pellet was washed three times with 80% ethanol and dried in the SpeedVac. The starch was converted to glucose by adding an α-amylase solution (1mg/ml in water, Rohalase® A3 from *Bacillus subtilis*, 44 U/mg, Serva) and heating the solution to 90ºC in a shaking water bath and subsequently adding an amyloglucosidase solution (0.5 mg/ml in 50mM citrate buffer, pH 4.6, amyloglucosidase from *Aspergillus niger*, 70U/mg, Sigma) and shaking it at 60 ºC. After centrifugation, the supernatant was diluted 0-50 times depending on the initial sample size. Glucose levels were analyzed with the HPIC, which this time was eluted with 100 mM NaOH + 25 mM sodium acetate.

Gene expression analysis

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to determine gene expression. Frozen leaf material was ground until fine and kept frozen for RNA extraction with the RNeasy plant minikit (Qiagen). RNA concentration and quality was tested with a spectrophotometer (NanoDrop, Thermoscientific, Thermofisher). 1 µg of RNA was used for DNAse treatment using Amplification grade DNAse I (Invitrogen, Thermofisher). RNA was synthesized to cDNA with the iScript kit (Bio-Rad). RNA extraction, DNase treatment and cDNA synthesis were performed as described in the manufacturers' protocols. 20 µl of synthesized cDNA was diluted with water to a total volume of 150 µl. 5 µl of SYBR-green (iQ-SYBR-green super mix, Bio-Rad), 0.25 µl Forward Primer (10 µM), 0.25 µl Reverse Primer (10 µM), 0.5 µl Milli-Q

water and 4 µl diluted cDNA were used for the qPCR. Three technical replicates were used per sample and a water control (no cDNA) was done per primer pair. The samples were placed in a Thermal Cycler (C1000, Bio-Rad) that was set to 95°C for 3 min, then 40 cycles of 95°C for 10 sec and 58°C for 60 sec, and then 95°C for 10 sec, and for the melt curve an increase from 65°C to 95°C with steps of 0.5°C for 5 sec.

The primers used for *StSP3D* (scaffold PGSC0003DMB00000014, unannotated) were (F) GGACCCAGATGCTCCAAGTC, (R) CTTGCCAAAACTTGAA-CCTG. For *StTPS1* (PGSC0003DMT400045229) the primers were (F) GCTTGTGC-AGGGATCCAAAG, (R) GGCATCATGATTTGGCCTCAC. The reference gene was *StNAC* (nascent polypeptide-associated complex alpha, PGSC0003DMT4000722-20) and the primers were (F) ATATAGAGCTGGTGATGACT, (R) TCCATGATAGC-AGAGACTA.

Statistical analysis

For the flower bud appearance time and the number of leaves formed before the inflorescence in CE3027, Exp. 1 and the two rounds of Exp. 2 were considered as three repetitions. The experiment number was considered a block effect and was added to a one-way analysis of variance (ANOVA) (main effects: "light treatment" and "experiment"). Pairwise comparisons were done with a Fisher's protected LSD ($\alpha = 0.05$). For flowering time in *S. andigena,* the two repetitions in Exp. 2 were considered and an ANOVA and Fisher's protected LSD ($\alpha = 0.05$) were used. The same was done for all other comparisons. When the data was not normally distributed (tested with a Shapiro-Wilk W-test for non-normality) a non-parametric Kruskal-Wallis test and Dunn's pairwise comparison ($\alpha = 0.05$) were used. In some cases there were no real repetitions as experiments could not be grouped (sucrose and starch concentrations in Exp. 1 and flowering time in transgenic lines). In these cases biological replicates were considered as independent experimental units. The analyses were computed in SPSS (IBM, SPSS Statistics 22). For gene expression analysis of Exp. 2, plants from one climate chamber were used. Three technical replicates per pooled sample of three biological replicates were used for the qPCR analysis. To calculate relative gene expression compared to the reference gene, the following formula was used: $100/2^{-\Delta Ct}$. Ct (cycle threshold) values of the gene of interest (*StSP3D* and *StTPS1*) were expressed as a percentage of the reference gene *StNAC.*

Results

High DLI accelerates flowering

In both *S. andigena* and CE3027, an increase in DLI significantly accelerated flower bud appearance in both genotypes (Fig. 4.1A, C), with a more pronounced effect at the lower than at the higher DLIs. A doubling of DLI in the short-day

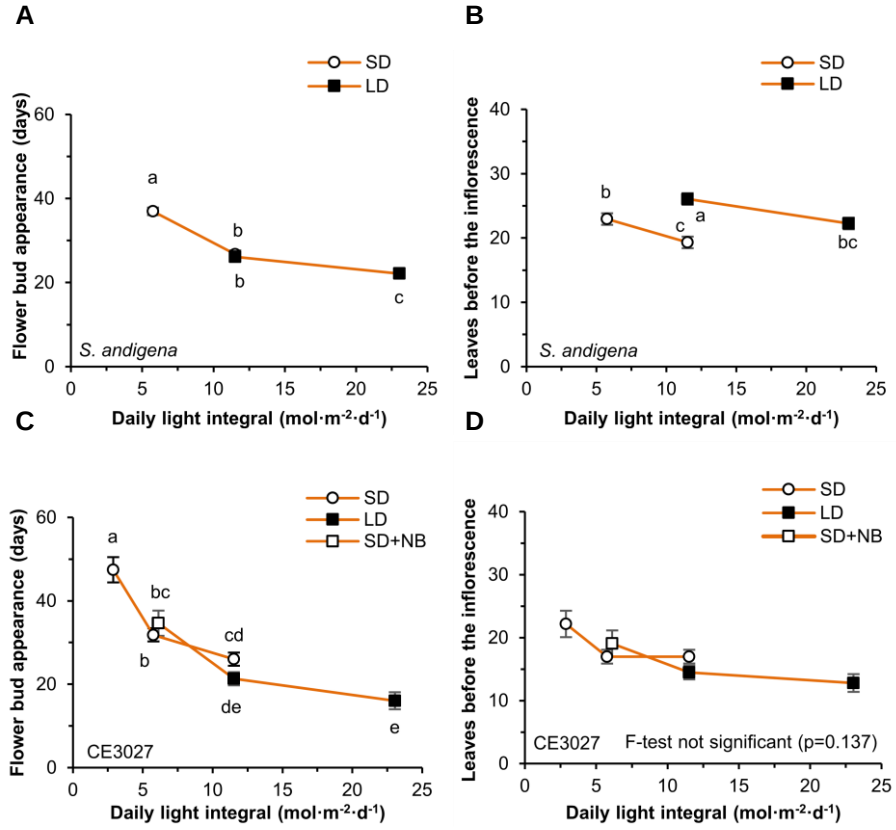


Figure 4.1. Flowering time in several light treatments. Time from transplanting until first flower bud appearance (A, C) and the number of leaves formed before flower bud formation (B, D) in *S. andigena* (A, B) and CE3027 (C, D). Plants were grown in different daily light integrals (DLI) in short days (SD, 8/16 hours light/dark, open circles), long days (LD, 16/8 hours light/dark, closed squares) and in a short days with a night break of 15 minutes in the middle of the night (SD+NB, 8/16 hours light/dark, open squares). Error bars are the standard error of difference (ANOVA) (when not visible, standard errors are smaller than the data markers). Different letters indicate significant differences between light treatments (Fisher's protected LSD test, $\alpha = 0.05$).

treatment, accelerated flower bud appearance by close to 10 days in *S. andigena* and 15 days in CE3027. The night-break treatment imposed on CE3027 plants was successful in delaying tuberization similarly to long days (data not shown), but resulted in a flowering time similar to the short day with the same DLI (Fig. 4.1C). Flowering time was also considered in the number of leaves formed prior to the appearance of the inflorescence. The number of leaves is a measure of the flower initiation time, considering no more leaves are formed on the primary stem after the inflorescence (Almekinders and Struik, 1996). In *S. andigena,* when comparing between long and short days, the difference in leaf number was clearer than the difference in days till flowering (Fig. 4.1B). Moreover, under both long and short-day conditions, an increase in DLI significantly decreased the number of leaves formed before the inflorescence and thus accelerated flower initiation. CE3027 plants grown in higher DLIs formed less leaves (Fig. 4.1D), but these differences were not significant.

Sucrose concentrations in shoot apex and leaves were increased under high DLIs, but only after flower buds appeared

To determine whether increased DLI affected flowering time through increased carbohydrates, we measured sucrose concentrations in the shoot apex and in the leaves. In the first experiment, where only CE3027 was tested, a significant increase in leaf and shoot apical sucrose was measured in the treatments with higher DLIs (Fig. 4.2A-B). However, this increase in sucrose concentration was only visible in plants that were growing for 42 days, long after the first flower buds had appeared (except in the short-day treatment with the lowest DLI). Furthermore, the shoot apices that were sampled after flower buds had formed, consisted of generative tissue while shoot apices in earlier sampling times were still vegetative, making comparisons in shoot apical material unreliable.

To validate that differences in sucrose levels under different light treatments only occurred after flower bud appearance, leaf tissue was sampled at different time points in the experiment testing both *S. andigena* and CE3027. In both short and long days in *S. andigena*, the treatments with the higher DLI (SD 11.5 and LD 23) had a higher sucrose concentration in the leaves early on (after 21 days of growing) (Fig. 4.3A). However, the sucrose concentrations did not correlate with the flowering time in days, as the SD 11.5 flowered at the same time as the LD 11.5, but had significantly higher sucrose concentrations.

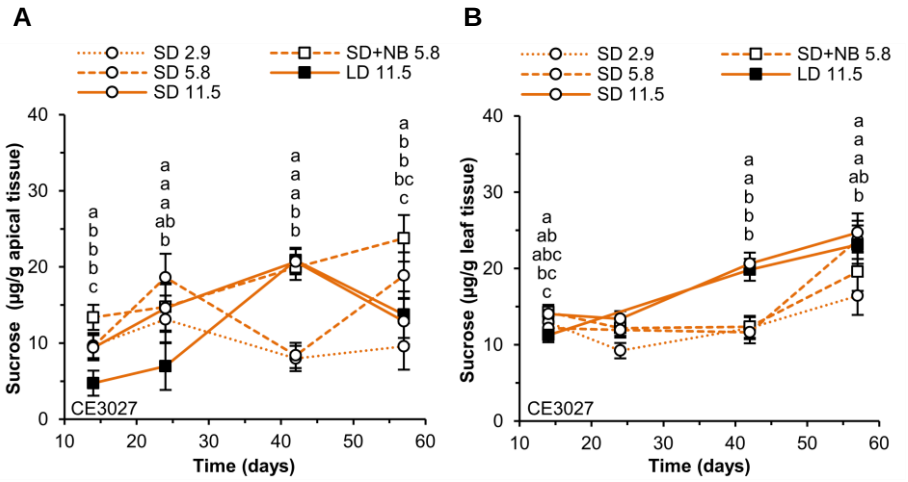


Figure 4.2. Sucrose concentrations in CE3027 under different daily light integrals (DLI). Sucrose concentration in the shoot apex (A) and in leaves (B) of CE3027 at four sampling times at different DLIs. Plants were grown in short days (SD, 8/16 hours light/dark, open circles), long days (LD, 16/8 hours light/dark, closed squares) and in a short days with a night break of 15 minutes in the middle of the night (SD+NB, 8/16 hours light/dark, open squares). Error bars show the standard error of difference (ANOVA). The numbers behind the day lengths are the DLIs in $mol \cdot m^{-2} \cdot d^{-1}$. Different letters indicate a significant difference (Fisher's protected LSD, $\alpha = 0.05$) between light treatment at a given sampling time. When no letters are shown, light treatments did not differ significantly. Dotted lines show the lowest DLI, dashed lines the intermediate DLI, and solid lines the highest DLI.

Additionally, CE3027 still did not show a significant difference in sucrose concentration until after flower bud appearance (Fig. 4.3B). At this later sampling time, leaf sucrose did not correlate with flower bud appearance time, similar to *S. andigena*. Treatments with high starch concentrations did not correspond to treatments with faster flowering time or only peaked after flower bud appearance (Appendix, Fig. S4.2A-D).

T6P is considered a proxy of sucrose status in the plant. We measured *StTPS1* expression, which is required for T6P synthesis. In *S. andigena* the expression increased with increasing DLI (Fig. 4.4A). At the sampling time just before the first flower buds were visible (21 days), the expression followed a similar pattern to the time to flower bud appearance. In CE3027, *StTPS1* expression after 21 days hardly differed between treatments (Fig. 4.4B). After 35 days, the day length had a more pronounced effect on *StTPS1* expression than the DLI; long days had substantially higher expression than short days, but an increase in DLI within short or long days did not increase *StTPS1* expression.

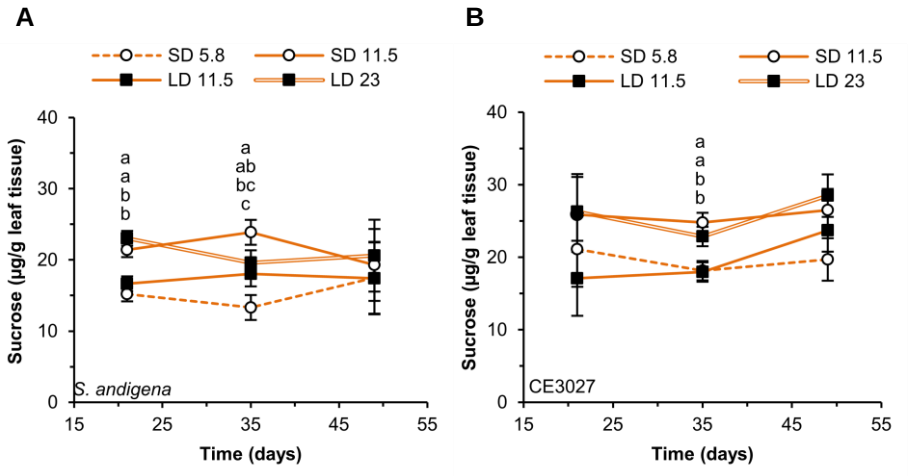


Figure 4.3. Sucrose concentrations in *S. andigena* and CE3027 under different daily light integrals (DLI). Sucrose concentration in the leaves of *S. andigena* (A) and CE3027 (B) at three sampling times in short days (SD, 8/16 hours light/dark, open circles) and long days (LD, 16/8 hours light/dark, closed squares) with different DLIs. The numbers behind the day lengths are DLIs in $mol \cdot m^{-2} \cdot d^{-1}$. Error bars show the standard error of difference (ANOVA). Different letters indicate a significant difference between light treatments at a given sampling time (Fisher's protected LSD, $\alpha = 0.05$). When no letters are given, differences between light treatments were not significant. Dashed lines show the lowest DLI, solid lines the intermediate DLI, and double lines the highest DLI.

StSP3D gene expression in short days is upregulated under high light

To find out if high DLIs would ultimately lead to a change in the *FT-like StSP3D*, we analyzed gene expression in *S. andigena* and CE3027 in short and long days in high and low DLIs. In short days an increase in DLI increased the expression of *StSP3D* after 35 days of growing, in both genotypes (Fig. 4.4C-D). However, in long days this increase was not visible and *StSP3D* expression remained extremely low. Expression was also tested in *S. andigena* after 21 days of growing, but the expression levels were very low and did not significantly differ between treatments (Appendix, Fig. S4.3).

Transgenic lines silenced in *StSP3D* flowered faster under high light

To determine if an increase in *StSP3D* expression under high light could explain accelerated flowering, we grew two *S. andigena* lines that were silenced in *StSP3D* in short and long days under low and high DLIs. The lines with decreased

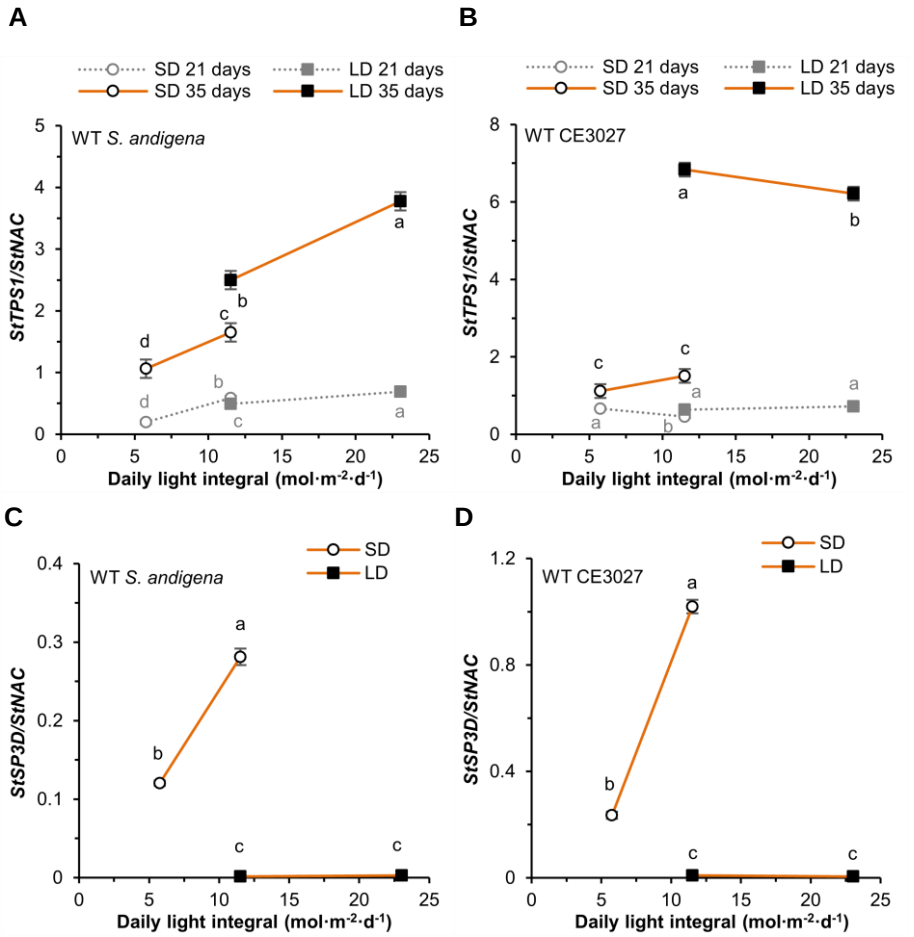


Figure 4.4. **Gene expression under different daily light integrals (DLI).** Gene expression of *StTPS1* (A-B) and *StSP3D* (C-D) in leaf tissue of *S. andigena* (A, C) and CE3027 (B, D) under different DLIs in short days (SD, 8/16 hours light/dark, open circles) and long days (LD, 16/8 hours light/dark, closed squares). *StTPS1* expression was measured in leaf material sampled after 21 and 35 days of growing. *StSP3D* expression was determined after 35 days of growing. Error bars show the standard error of difference of the ANOVA (when not visible, standard errors are smaller than the data markers). Identical letters signify no significant differences between light treatments within a sampling time (21 or 35 days, Fisher's protected LSD α= 0.05).

StSP3D expression flowered slightly later than the wild type (Fig. 4.5A-B). Like in the wild-type plants, the transgenic lines flowered significantly faster in the high light treatments, both in short and long days (with the exception of one transgenic line in which the leaf number was not significantly affected by high light in short days). Although flowering time was only slightly delayed in the

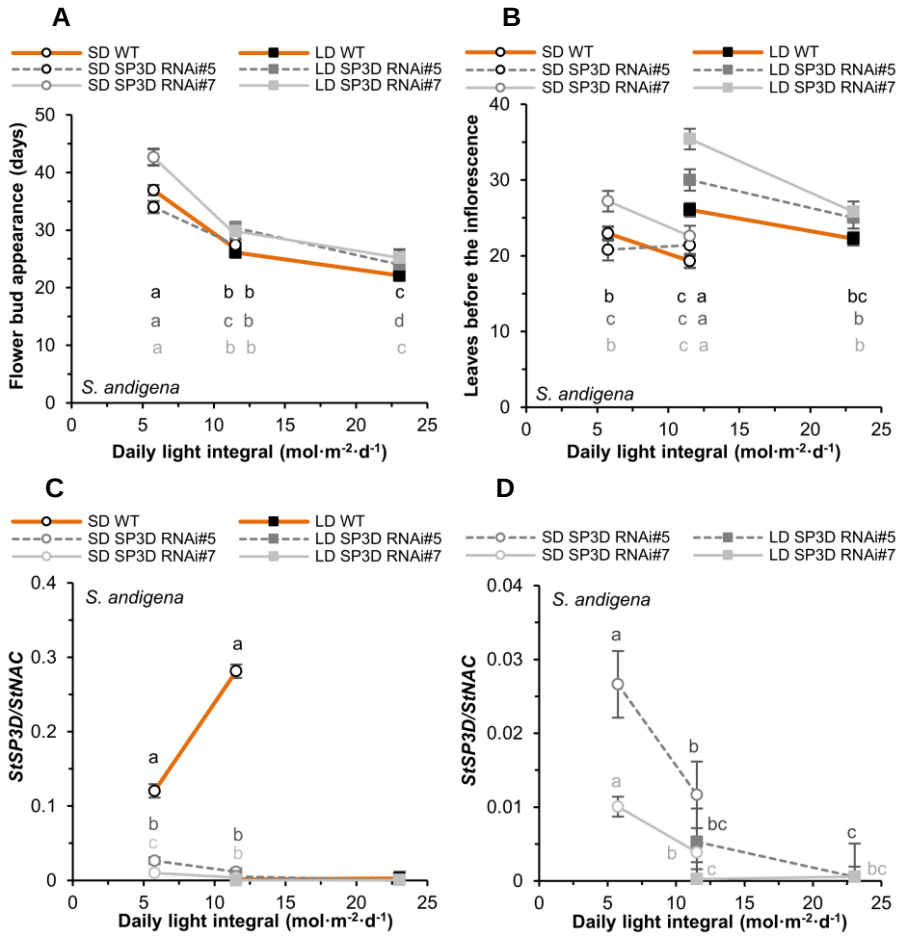


Figure 4.5. **Flowering time and gene expression in plants silenced in *StSP3D*.** Effect of the daily light integral (DLI) on days till flower bud appearance (A) and the number of leaves formed before flower bud appearance (B) in wild-type (WT) *S. andigena* and two lines silenced in *StSP3D*. The *StSP3D* expression is given for WT *S. andigena* and the *StSP3D RNAi* lines (C) and a detail of this expression is given showing only the transgenic lines (D). Plants were grown in varying DLIs in short days (SD, 8/16 hours light/dark, open circles) and long days (LD, 16/8 hours light/dark, closed squares). (A, B, D) Error bars indicate the standard error of difference (ANOVA) within a genotype between light treatments (when not visible, standard errors are smaller than the data markers). (C) Error bars show the standard error of difference within a light treatment between genotypes. Identical letters signify no significant difference between genotypes (C) or light treatments (A, B, D) (Fisher's protected LSD, $\alpha = 0.05$). Colors of the letters correspond to the genotypes. Where no letters are indicated, differences were not significant.

transgenic lines, *StSP3D* expression was strongly downregulated compared to the wild type (Fig. 4.5C). Furthermore, although very low, the remaining expression of *StSP3D* in the silenced lines decreased with increasing DLI,

showing an opposite pattern compared to the flowering phenotype (Fig. 4.5D). Thus acceleration of flowering under higher light was not paired with increased *StSP3D* levels.

Discussion

Flowering in potato is accelerated under high DLIs

Although a lot is known about the environmental control of flowering time in plants, environmental control of potato flowering is still largely unclear. Potato tuberization is under strong control of photoperiod, but the photoperiodic effect on potato flowering is less well defined. Although most potato genotypes initiate flowering under both long and short days, there is little consensus about the classification of potato as day-length neutral for flowering (Jones and Borthwick, 1938; Turner and Ewing, 1988; Almekinders and Struik, 1994, 1996; Martínez-García *et al.*, 2002; Schittenhelm *et al.*, 2004). Bernier *et al.* (1993) postulated that plants that do not need a particular day length to flower, can be sensitive to the amount of obtained light. Firman *et al.* (1991) stated that in potato, photoperiod has little influence on flower initiation time but low light levels had the ability to totally halt flowering. Our experiments show that an increase in DLI clearly accelerates potato flower bud appearance (Fig. 4.1). Although we measured flower bud appearance instead of flower initiation, which can only be determined destructively (Firman *et al.*, 1991), a high DLI also accelerated flower initiation. This was demonstrated by the reduced number of leaves formed before the inflorescence under high light.

Several studies indirectly determined the effect of the DLI on the number of leaves before the inflorescence, when extending the light period in day-length experiments (Almekinders, 1992; González-Schain *et al.*, 2012). The number of leaves before flowering was not affected in these studies. Our results also show the number of leaves before flowering is similar in long days with a high DLI (LD23) and in short days with an intermediate DLI (SD11.5) (Fig. 4.1B). However, these comparisons take into account both the DLI effect and the day-length effect. When only comparing between a low and high DLI in one day length, there is a clear decrease in the number of leaves formed before the inflorescence when the DLI was increased. While when comparing between a

short and long day with the same DLI, the plants in a short day formed less leaves (Fig. 4.1B). Perhaps a similar number of leaves is formed before the inflorescence in LD23 and SD11.5 because a high DLI leads to faster flower initiation (less leaves before the inflorescence) but a shorter day length also reduces the number of leaves formed before the inflorescence. Thus, not only a high DLI stimulates flower initiation, but shorter day lengths do as well. An effect of day length on the leaf number was only found in *S. andigena* and not in CE3027, thus this day-length effect may be genotype specific. Interestingly, the effect of day length on flower bud appearance time was minimal. In both CE3027 and *S. andigena* there was no difference in flower bud appearance time in short or long days when given the same DLI. This is in stark contrast to potato tuberization, which is induced by short days (Ewing and Struik, 1992).

It cannot be ruled out that light intensity, rather than the DLI, plays a role in flowering time. An increase in light intensity automatically means an increase in DLI within the same day length. By comparing between day lengths, the effect of either DLI or light intensity can be determined, but day length may also have an effect. However, our results suggest the DLI is the controlling factor. In a short day with a night break (sensed by the plant as a long day), plants flowered significantly slower than in long days with the same light intensity but double the DLI (Fig. 4.1C and Table 4.1), suggesting that not a higher light intensity but a higher DLI accelerates flowering.

The carbohydrate status in the potato plant does not correlate with flowering time

In several species, including potato, an increase in shoot apical sucrose was found preceding flowering (Eriksson *et al.*, 2006; Chincinska *et al.*, 2008). Although CE3027 in treatments with a higher DLI did have higher sucrose levels in the shoot apex (Fig. 4.2A), this increase happened after flower bud appearance. Also, when looking at leaf sucrose concentration (Fig. 4.2B), differences were only found after flower bud appearance. In *S. andigena*, leaf sucrose concentrations were higher in the short and long day with high light just before the first flower buds were visible. However, if the amount of leaf sucrose would be decisive for flowering time, the low-light long day should have the same sucrose concentrations as the high-light short day, which was not the case. Furthermore, leaf sucrose may not be very representative in potato, as in short

days a lot of the carbohydrates are mobilized from the leaves and transported to the tubers (Hannapel, 2007). Our results do not show a straightforward correlation between an increase in leaf and shoot apical sucrose concentrations and flower bud appearance time. However, a role of carbohydrates cannot be ruled out. Perhaps sampling of the whole shoot apex did not allow for the detection of sucrose increase in the shoot apical meristem, as was done in Chincinska *et al.* (2008). Earlier experiments in *Sinapis alba* describe a transient, but dramatic increase of the sucrose flux in apical phloem sap preceding floral transition (Lejeune *et al.*, 1993), which is believed to be derived from the mobilization of stored leaf carbohydrates (Levy and Dean, 1998). Perhaps more frequent sampling is needed to observe a transient increase in sucrose flux. Additionally, exogenous sucrose application may determine whether potato flowering can be accelerated through an increase in available sucrose.

Although we did not see a clear correlation between sugars and flower bud appearance, the sugar signaling may take place in different tissues or at different times than were measured. In this case, targets of sugar signaling could be an easier way to measure differences and may demonstrate how high light is accelerating potato flowering. T6P acts as a proxy for sucrose levels in the plant (Lunn *et al.*, 2006; Matsoukas *et al.*, 2012; Bolouri Moghaddam and Van den Ende, 2013). In Arabidopsis expression of *TPS1*, the gene responsible for the synthesis of T6P, is necessary for the transition to flowering (Wahl *et al.*, 2013). Interestingly, just before the first flower bud appearance in *S. andigena*, *StTPS1* expression in the leaves is affected by the light treatments in the same way flower bud appearance time was (higher expression under higher DLIs). However, in CE3027, *StTPS1* expression was not correlated with the DLI. More experiments are needed to clearly determinate the role of carbohydrates and T6P in DLI-mediated flowering time in potato.

StSP3D expression is increased under high light but acceleration of flowering by high light is not caused by an upregulation of *StSP3D*

The sugar mediated flowering pathway is known to act on *FT* expression (Bouché *et al.*, 2016). In potato, *StSP3D* was suggested as a possible flowering *FT*, considering *StSP3D* is a homolog of Arabidopsis *FT* and tomato *SINGLE FLOWER TRUSS,* and downregulation of this gene delayed flowering time (Lifschitz *et al*., 2006; Abelenda *et al.,* 2014; Navarro *et al.*, 2011). We determined whether

StSP3D leaf expression was affected by the DLI. In both *S. andigena* and CE3027, *StSP3D* expression was upregulated under high light in short days. Since flowering was accelerated in short days under high light, *StSP3D* expression may mediate flowering time. However, *StSP3D* expression was extremely low in the long-day treatments and was not increased under high light. In long days, a second potato *FT*-homolog called *StSP5G* represses expression of a third *FT*-homolog *StSP6A*, which encodes for the tuberization signal (Abelenda *et al.,* 2016). Perhaps high light promotes *StSP3D* expression in both short and long days, but StSP5G also represses *StSP3D* in long days, as is the case in tomato (Soyk *et al.*, 2017). Nevertheless, the low long-day expression levels were not in line with the flowering time, since long-day plants flowered similarly to short-day plants when given the same DLI.

These results imply that *StSP3D* leaf expression does not correlate with flowering time. Several alternatives may explain this lack of correlation. Although StSP3D has been shown to be crucial in potato flowering (Navarro *et al.*, 2011), *StSP3D* leaf expression may not control potato flowering. In onion, which like potato has a dual reproduction strategy, bulb formation is induced by a mobile signal formed after photoperiodic induction of *AcFT1* in the leaves, as is the case for tuberization (Lee *et al.,* 2013). However, flower induction in onion is induced through *AcFT2* expression in the bulb meristematic tissue (Lee *et al.*, 2013). The authors propose, that because *AcFT2* is expressed in the tissue where it acts, AcFT2 may not need transporting from the leaves. A similar mechanism may take place in potato flowering, making leaf *StSP3D* expression unnecessary for successful flower induction. Another possibility is that potato flowering is regulated by more *FTs* and that only short-day flowering is regulated by *StSP3D*. The FT family in Solanaceae have undergone considerable expansion (Abelenda *et al.,* 2014), making involvement of additional FTs in potato flowering plausible. Combined FT regulation on flowering has been documented before. For example, three *FTs* were found to regulate Chrysanthemum flowering; one regulated flowering in short days, a second regulated flowering in long days and a third regulated flowering in response to sugar levels in the plant (Sun *et al.*, 2017).

Considering that *StSP3D* leaf expression does not correlate with flowering time, it is unlikely for high-light accelerated flowering to be controlled through *StSP3D* expression in the leaves. However, high light did upregulate leaf *StSP3D* in short days, therefore this experiment did not yet rule out that in short days, accelerated flowering is caused by upregulated *StSP3D*.

To determine whether accelerated flowering under high light could be caused by the upregulation of *StSP3D,* we grew two *S. andigena* transgenic lines silenced in *StSP3D* under low and high light conditions. These lines had significantly reduced *StSP3D* expression (Fig. 4.5C), but still had accelerated flowering time under high light conditions (Fig. 4.5A-B), strongly indicating that high-light accelerated flowering is not controlled through the upregulation of *StSP3D*. The flower bud appearance in the *StSP3D* silenced lines mimicked the wild-type pattern, with increasing DLI. Furthermore, leaf *StSP3D* expression in the *StSP3D* silenced lines, although extremely low, was not upregulated in the high light treatments in long or short days. This finding rules out that a remaining low-level *StSP3D* upregulation may have caused high-light accelerated flowering. As silencing reduces *StSP3D* in all plant tissues, it seems that *StSP3D* in the apical meristem also cannot explain high-light accelerated flowering. However, it should be determined how *StSP3D* is expressed in the apical meristem in silenced lines, to rule out this possibility.

Unexpectedly, the lines silenced in *StSP3D* did not flower much later than the wild-type lines, even though *StSP3D* expression levels were significantly lower than in the wild type. This finding, in addition to the low expression of leaf *StSP3D* in long days, suggests that StSP3D is not the major factor controlling potato flowering time. However, silenced lines still retain some *StSP3D* expression (similar to long-day wild-type levels). Therefore determining if fully knocking out *StSP3D* expression with techniques like CRISPR/Cas9 (Wang *et al.*, 2015) totally inhibits flower initiation, may elucidate the contribution of *StSP3D* to potato flowering. Regardless, our results show that flowering time under high DLIs is accelerated, but this acceleration is not controlled through an upregulation of leaf *StSP3D*.

Possible ways high light accelerates flowering independently of *StSP3D* upregulation

If the DLI does not control flowering through altered leaf *StSP3D* expression, how then? We did not find a correlation between carbohydrate content and flowering, although *StTPS1* expression in *S. andigena* is increased in higher DLIs. It has been proposed that assimilates influence flowering indirectly, by controlling the mass flow of the phloem sap which transports necessary substances for flowering (like *FT*) to the shoot apical meristem (Bernier and Périlleux, 2005). This would mean *StSP3D* expression does not have to change for an accelerated flowering.

Alternatively, independent expression of *StSP3D* in the shoot apical meristem may explain floral transition, avoiding the need for phloem based transport for induction of downstream floral genes. However, the question remains how high light fits into this control. As mentioned before, a peak in shoot apical sucrose precedes flowering (Eriksson *et al.*, 2006; Chincinska *et al.*, 2008). If *StSP3D* is expressed in the apical meristem, sucrose may act on this *StSP3D* through a pathway similar to sucrose-induced *FT* expression in Arabidopsis (Bouché *et al.*, 2016). However, *StSP3D RNAi* lines, where shoot apical meristem *StSP3D* should also be down regulated, still had accelerated flowering under high DLIs, indicating shoot apical *StSP3D* may not play a role.

Instead, sucrose may act on downstream targets of *StSP3D* in the shoot apical meristem, which has also been found to happen in Arabidopsis with *FT* (Eriksson *et al.*, 2006). Downstream assimilate action on flowering is strengthened by the hypothesis that FTs StSP3D and StSP6A (tuberization signal) may actively redirect the assimilate flow to either flowering or tuberization, respectively, to regulate these processes (personal communication Abelenda, 2018). In this case StSP3D may affect assimilates, instead of the other way around. Nevertheless, it must be ascertained if sugars play a role in high-light accelerated flowering time.

Conclusion

We conclude that high DLIs accelerate flower initiation in potato. This accelerated flowering is controlled independently of *StSP3D* upregulation, the proposed flowering *FT* in potato.

Acknowledgements

This research is part of the FlowerPot project which was supported by the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO), Signify, Solynta, Beekenkamp Plants and Incotec. The funding sources had no involvement in the study design, data collection, analysis or writing of the paper. We thank Signify for providing the LEDs for this project. Special thanks to Joep Debets for the extensive data collection during his bachelor thesis project.

Appendix

Supplementary text 1. To determine peak *StSP3D* expression throughout the day, gene expression analysis was performed in plants growing in short and long days. *S. andigena* wild-type plantlets were transplanted from tissue culture to pots (7 x 7 x 8 cm) and grown in a climate chamber in short days with eight hours of 300 µmol·m^{-2}·s^{-1} light and long days with 16 hours of 150 µmol·m^{-2}·s^{-1} light. The light treatments were light-tight and separated from each other. Other climate conditions were the same as in Exp. 1 described in the Materials and Methods. After four weeks of growing on soil, leaves were sampled for gene expression analysis. Per time point the fourth and fifth leaf from the shoot apex were sampled from three plants. These leaf samples were pooled into one sample per time point and treatment. Samples were taken for a period of 40 hours at ZT (Zeitgeber time, hours after start of light period): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 1, 3, 5, 7, 9, 11, 13, and 15. After sampling, leaves were frozen in liquid nitrogen and stored at -80ºC. RT-qPCR was done to determine gene expression and was performed as described in the Materials and Methods. The reference gene used for these analyses was *StEIF3e* (PGSC0003DMT400076704, *EUKARYOTIC INITIATION FACTOR 3E SUBUNIT*), which has been used before in potato as a reference gene in 24-hour time courses (Kloosterman *et al.*, 2013). Primers were (F) GGAGCACAGGAGAAGATGAAGGAG, (R) CGTTGGTGAATGCGGC-AGTAGG. *StSP3D* expression is given in Fig. S4.1.

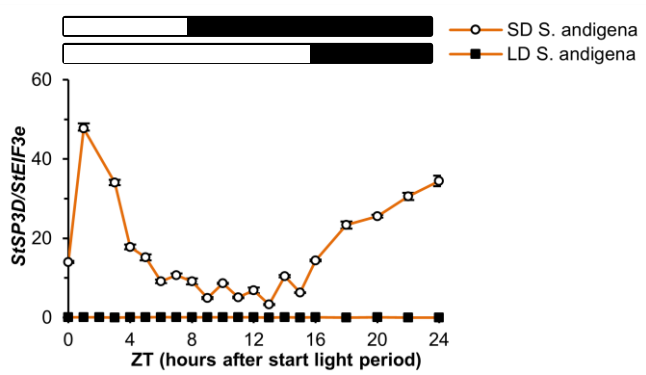


Figure S4.1. Time course of *StSP3D* expression in *S. andigena*. Plants were grown in short days (SD) of 300 µmol·m^{-2}·s^{-1} and long days (LD) of 150 µmol·m^{-2}·s^{-1}.

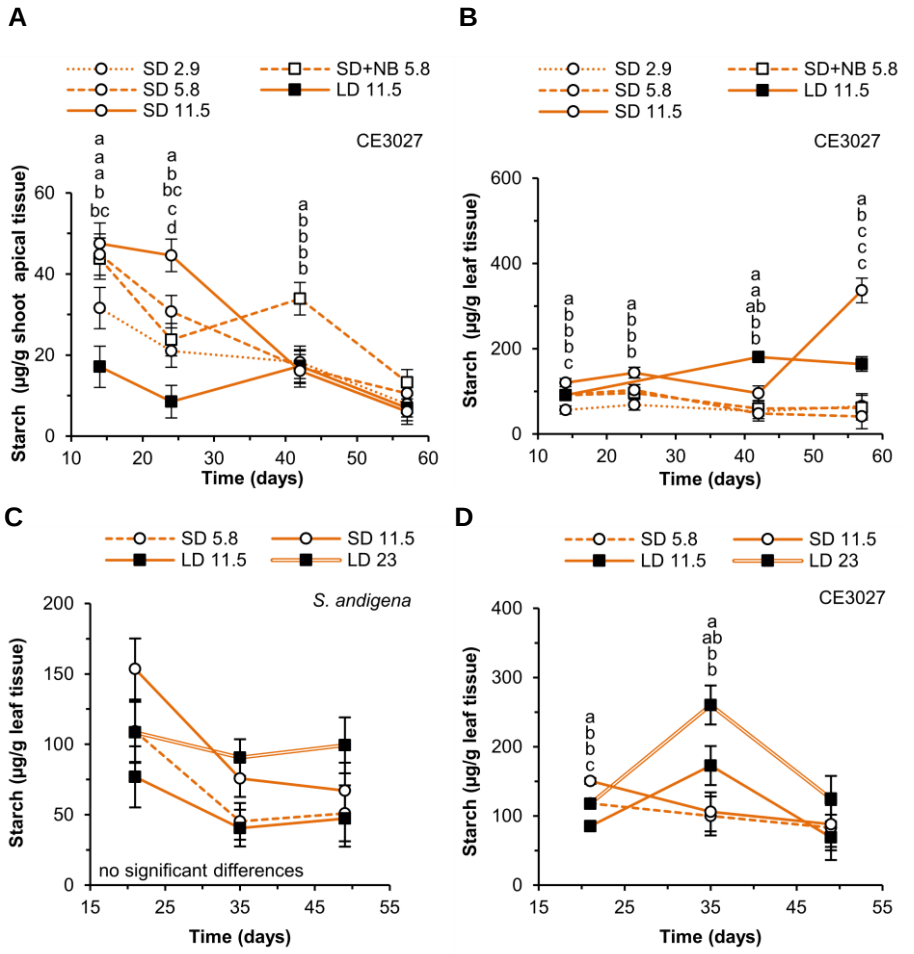


Figure S4.2. Starch concentrations in shoot apical and leaf tissue in CE3027 and *S. andigena*. Starch in shoot apex (A) and leaf (B) in experiment 1 with CE3027. Starch in leaves of S. andigena (C) and CE3027 (D) in experiment 2. SD (short day, 8/16 hours light/dark, open circles), LD (long day, 16/8 hours light/dark, closed squares), NB (night break of 15 min in the middle of the night, short day, 8/16 hours light/dark, open squares). Error bars show the standard error of difference (ANOVA). The numbers behind the day lengths are the daily light integrals (DLI) in $mol \cdot m^{-2} \cdot d^{-1}$. Different letters indicate a significant difference (Fisher's protected LSD, $\alpha = 0.05$) between light treatment at a given sampling time. When no letters are given, there are no significant differences. Dotted lines show the lowest DLI, dashed lines the intermediate DLI, and solid lines the highest DLI (A-B). In C-D an additional higher DLI is added, which is depicted with a double line.

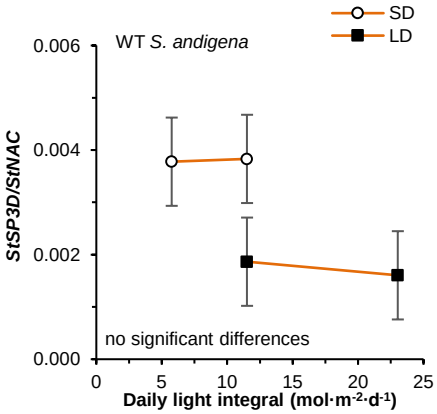


Figure S4.3. *StSP3D* expression in *S. andigena* under different daily light integrals (DLI). Plants were grown in short days (SD, 8/16 hours light/dark, open circles) and in long days (LD, 16/8 hours light/dark, closed squares). Leaf material was sampled 21 days after transplanting to soil. Error bars show the standard error of difference of the ANOVA. No Significant differences were found between treatments.

5. The tuberization signal StSP6A represses flower bud development in potato

Faline Plantenga, Sara Bergonzi, José Abelenda, Christian Bachem, Richard Visser, Ep Heuvelink and Leo Marcelis

A revised version was accepted for publication in Journal of Experimental Botany, 2018, DOI: 10.1093/jxb/ery420

Abstract

Potato (*Solanum tuberosum* L.) can reproduce sexually through flowering and asexually through tuberization. While tuberization has been thoroughly studied, little research has been done on potato flowering. Flower bud development in the strictly short-day tuberizing *Solanum tuberosum* L. ssp. *andigena* is impaired under short-day conditions. This impaired development may either indicate that day length regulates flowering oppositely to tuberization, that tuberization negatively influences flowering, or both. The aim of this research was to determine how tuberization affects flower bud development. To find out whether the absence of tubers improves flowering we prevented tuberization by: (1) grafting potato scions onto wild potato rootstocks, which were unable to form tubers; (2) removing stolons, the underground structures on which tubers form; (3) using plants that were silenced in the tuberization signal *StSP6A*. The absence of a tuber sink alone did not accelerate flower bud development, nor did it allow more plants to reach anthesis (open flowering stage) or have more open flowers. Interestingly, reducing *StSP6A* expression by gene silencing did improve flower bud development. Our results show that flower bud development in potato is repressed by the tuberization signal StSP6A, and not by competition with the underground tuber sink.

Keywords: day length, grafting, potato flowering, stolons, StSP6A, tuberization

Introduction

Potato (*Solanum tuberosum* L.*)* is the third largest crop for human consumption worldwide and due to its high nutritional value and low production costs, consumption is most certainly expected to increase (International Potato Center, 2016; Zaheer and Akhtar, 2016). Potato plants are able to reproduce both sexually, through flowers, and asexually through the formation of tubers. Although both reproduction methods are present in the plant, most research has been done on tuberization. Commercial potato production mainly uses "seed tubers" and not "true seeds" to propagate plants. Asexual reproduction is used for propagation because potato plants are tetraploid and highly heterozygous. Incorporation of a *Sli* gene allows for self-fertilization of diploid potato lines, which makes the generation of homozygous lines possible (Lindhout *et al*., 2011). These developments have made hybrid breeding possible in potato and thereby also the use of true potato seeds as starting material. Hybrid breeding of potato will enable breeders to specifically select for desired traits in new varieties and develop these varieties much faster than in traditional potato breeding (Lindhout *et al*., 2011). The developments in potato breeding and propagation require the understanding of not only tuberization, but also potato flowering.

Whether a potato plant starts to tuberize or flower, depends strongly on environmental cues (Ewing and Struik, 1992; Almekinders and Struik, 1996). Potato tuberization is strongly influenced by day length and is induced under short-day conditions (Batutis and Ewing, 1982). Modern varieties are no longer dependent on short days to tuberize, as breeders have selected against this trait. Nevertheless, the photoperiodic mechanism controlling tuberization remains conserved in all potato plants (Kloosterman *et al*., 2013). As potato tuberization has been intensively studied, we have a good understanding of the molecular regulation behind this process (Abelenda *et al.* 2011; Navarro *et al.*, 2011; González-Schain *et al.* 2012; Navarro *et al.*, 2015). The photoperiodic regulation of tuberization strongly resembles the photoperiodic control of flowering time in the model plant *Arabidopsis thaliana* and other plants (Tsuji *et al.,* 2011; Andrés and Coupland, 2012; Fu *et al.*, 2014). *SELF-PRUNING 6A* (*StSP6A*) was identified as a potato homolog of the flowering signal *FLOWERING LOCUS T* (*FT*) in *A. thaliana* and instead of inducing the flower transition, StSP6A induces tuber formation in potato (Potato Genome Sequencing Consortium, 2011; Navarro *et*

al., 2011). After *StSP6A* is expressed in the leaves, the mobile StSP6A protein moves through the plant to underground stems, called stolons, where it induces tuberization. The cascade of events leading to short-day dependent expression of *StSP6A* mRNA has also been revealed. This control includes genes such as *CYCLING DOF FACTOR 1* (*StCDF1*) and *CONSTANS* (*StCOL1*) (Kloosterman *et al*., 2013), which are also involved in photoperiodic control of flowering in *A. thaliana*. In potato, StCDF1 downregulates *StCOL1*, which in turn induces *SELF-PRUNING 5G* (*StSP5G),* a repressor of *StSP6A* (Kloosterman *et al*., 2013; Abelenda *et al.*, 2016). Within the Solanaceae, the *FT* family has undergone a large expansion and another homolog of *FT* called *SELF-PRUNING 3D* (*StSP3D*) was found in potato and was proposed to control the flower transition (Potato Genome Sequencing Consortium, 2011; Navarro *et al.*, 2011). However, how this regulation takes place remains to be elucidated.

Although some research has been performed on potato flowering, ambiguity remains concerning the environmental effect on flower initiation and whether this is a long-day, short-day or day-neutral process (Jones and Borthwick, 1938; Almekinders and Struik, 1994; Navarro *et al.*, 2011; González-Schain *et al.*, 2012). Although little is known about flower initiation, it has been established that potato flower development is negatively affected in tuber inducing conditions like short days (Turner and Ewing, 1988; Rodríguez-Falcón *et al.*, 2006; Plantenga *et al*., 2016). Flower buds abort more frequently and less open flowers are formed. Failure of flower bud development in short days could be due to a direct photoperiod effect, but alternatively might be the result of a negative effect exerted by tuberization. Tubers are strong assimilate sinks (Sweetlove *et al.,* 1998) and may leave insufficient assimilates to support flowering (Almekinders and Struik, 1996). However, previous studies do not agree whether or not flowering competes with tuberization (Krantz, 1939; Thijn, 1954; Jessup, 1958; Krauss and Marschner, 1984; Pallais, 1987).

Here we confirm that while flower initiation occurs independently of the photoperiod, later stages of flower bud development are impaired under short-day conditions which induce tuberization. Specifically, we investigated whether flower bud development is impeded by competition for assimilates between flowering and tuberization or by the tuberization signal StSP6A. We performed experiments where we prevented tuberization in three different ways; (1) by grafting potato scions onto wild potato rootstocks, that were unable to form tubers; (2) by removing stolons, the structures on which tubers form; (3) by using transgenic plants that were silenced in the tuberization signal *StSP6A* (Fig. 5.1). Finally we demonstrated how increased

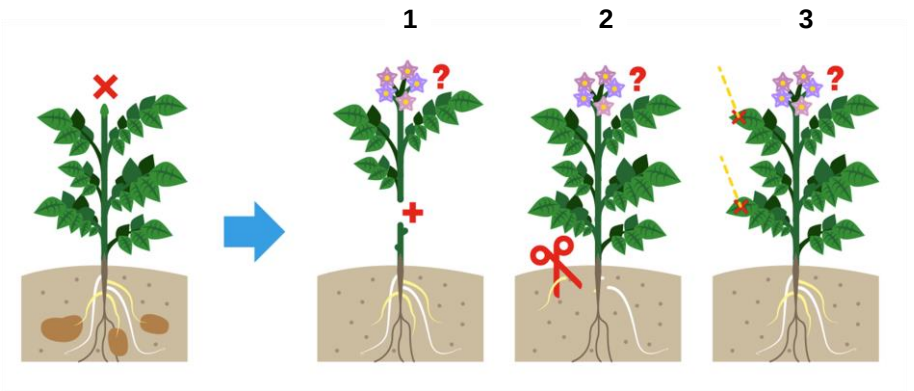


Figure 5.1. The three methods used to eliminate tuberization in potato and determine whether flower bud development is improved. (1) Scions of potato plants that are able to tuberize are grafted onto non-tuberizing wild potato rootstocks (2) The stolons of the potato plant are removed as soon as they appear. (3) The tuberization signal StSP6A, which is expressed in the leaves, is silenced in transgenic lines.

StSP6A expression affected flower bud development in long days. Together, our experiments show that the tuberization signal StSP6A represses flower bud development.

Materials and methods

Plant materials

Solanum tuberosum L. ssp. *andigena* (*S. andigena*), *Solanum tuberosum* L. ssp. *tuberosum* (*S. tuberosum*) CE3027 and *S. tuberosum* CE3130 were used. *S. andigena* is a tetraploid, obligate short-day plant for tuberization. CE3027 and CE3130 are progeny plants from a mapping population that segregates for timing of tuberization (Kloosterman *et al*., 2013), where CE3027 tuberizes early in short days and late in long days, and CE3130 tuberizes early under both short and long days. These diploid lines were used because they can produce open flowers in our climate chamber conditions, as opposed to *S. andigena*. All genotypes were propagated *in vitro* and maintained in tissue culture in MS20 medium (Murashige and Skoog, 1962). Additionally, two wild *Solanum* species that are unable to tuberize were used: *Solanum etuberosum* (*S. etuberosum*) (CGN17714) and *Solanum palustre* (*S. palustre*) (CGN18241) (CGN seedbank, Wageningen, Netherlands). Seeds of these species were disinfected in 2.7%

NaOCl for 30 minutes, soaked in 700 ppm gibberellic acid (GA_3) for 24 hours in the dark and sown on MS20. Finally, two *StSP6A* silenced lines in an *S. andigena* background (*StSP6A RNAi #1* and *StSP6A RNAi #13*) and two *StCDF1* over-expressing lines in a CE3027 background (*35S::StCDF1#3 and 35S::StCDF1 #4)* were used.

Plant transformation

In order to generate these lines, *StSP6A* coding regions were PCR amplified from *S. andigena* cDNA through Phusion High-Fidelity DNA Polymerase (Thermo Scientific™) using specific primers (RNAi6Afor 5'-CACCTACAAATACAAGCTTTG-GAA-3' and RNAi6Arev 5'-CTCTATTTATTTATAAC-AT-3'). They were then cloned in pENTR™/D-TOPO® (Invitrogen) following manufacturer recommendations. The final *StSP6A RNAi* construct was generated using the *StSP6A* pENTR™/D-TOPO entry clone and further insertion by recombination with the LR clonase™ II enzyme (Invitrogen) into the pK7GWIWG2(II) vector (Karimi *et al.*, 2002). Transgenic plants bearing the *StSP6A RNAi* construct were generated by *Agrobacterium*-mediated transformation of *in vitro* internodes as described previously in Visser (1991) .

The StCDF1.1 coding region was also amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific™) from *S. andigena* cDNA (same primers as for RNAi) and cloned in pENTR™/D-TOPO® (Invitrogen) as previously described (Kloosterman *et al*., 2013). Binary plasmids were obtained after LR clonase™ II enzyme (Invitrogen) reaction of StCDF1.1-pENTR™/D-TOPO® with the pK7WG2 plasmid, obtaining the 35S::StCDF1.1 plasmid (Karimi *et al.*, 2002). In order to generate 35S::StCDF1 transgenic plants, *Agrobacterium*-mediated transformation of CE3027 internodes with both plasmids was performed as described in Visser (1991).

S. andigena StSP6A RNAi and CE3027 *35S::StCDF1* plantlets were propagated *in vitro* and grown with the other potato plants.

Growing conditions and measurements

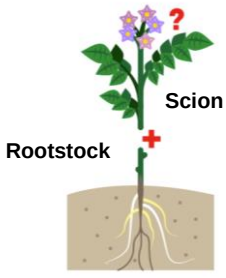
Experiment 1. Removing the tuber sink: grafting onto a non-tuberizing rootstock
Two grafting experiments were performed in a greenhouse in short-day conditions and in long-day conditions. In short days CE3027 scions were grafted

onto *S. etuberosum* and *S. palustre* rootstocks and vice versa. Also control grafts were made where scions were grafted onto rootstocks of their own genotype. In long days, nine grafting combinations were made between CE3027, CE3130 and *S. etuberosum* (Table 5.1). Additionally, grafts were made where CE3027 and CE3130 rootstocks maintained their leaves to ensure the production of the tuberization signal StSP6A. Details of the experiment can be found in the Appendix, Table S5.1.

In vitro plantlets were transplanted to 5 L pots with a clay-peat mixture. Grafting was done with two week old CE3027 and CE3130 plants and three week old *S. etuberosum* and *S. palustre* plants. The stem was cut approximately after the fourth leaf from the bottom. A splice graft was made and the rootstock and scion were kept together with silicone grafting clips (Beekenkamp 1.5 mm and Simonetti 2.9 mm). Leaves were removed from the rootstock, unless indicated otherwise. Grafts were placed in a high humidity compartment until the grafting unions had set. The plants were manually watered and fertilized ($2g \cdot L^{-1}$, Osmocote Exact Standard 3-4M, Everris). Flowering and tuberization were determined once a week. Anthesis (opening flowering stage) and the number of flowers per plant were determined visually by checking the shoot apex. Tuberization time was determined by removing soil around stem and stolons and checking for swelling of the stolon tip. Nine weeks after grafting, the tubers were harvested, oven-dried at 105ºC and weighed.

Table 5.1. Grafting combinations in long days.

Scion	Rootstock	Number of grafts (*n*)
S. etuberosum	*S. etuberosum*	3
CE3027*	CE3027	11
CE3027	*S. etuberosum*	8
S. etuberosum	CE3027	9
S. etuberosum	CE3027 (with leaves)	10
CE3130	CE3130	10
CE3130	*S. etuberosum*	9
S. etuberosum	CE3130	11
S. etuberosum	CE3130 (with leaves)	12



*CE3027 and CE3130 are two tuberizing potato genotypes and *S. etuberosum* is a wild non-tuberizing *Solanum* species.

Experiment 2. Removing the tuber sink: removing stolons

CE3027 plantlets were transplanted to 17cm Ø pots filled with a clay-peat mixture and placed in a climate chamber (details can be found in the Appendix, Table S5.1). Plants were grown in short days (8 hours light) under 200 or 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (photosynthetic photon flux density) light (SD200 and SD400 respectively) and in long days (16 hours light) under 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light (LD200). The high-light short day and low-light long day received the same daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$). In half of the plants in each light treatment, stolons were removed as soon as formed, resulting in six treatments in total. Light emitting diodes (LEDs) were used for the lighting (Philips GreenPower LED production module 120 cm DeepRed/White-2012). Light intensities were measured at the top of the plant canopy with a quantum sensor (LI-COR Biosciences, LI-190SB Quantum, LI-1400 data logger) and corrected by adjusting LED height every two weeks. Plants were rotated three times a week to ensure a homogenous light distribution. Side shoots were removed. Water was given manually and liquid fertilizer was supplied once per week (EC 2.1 dS m^{-1}, pH 5.5; 1.2 mM NH_4^+, 7.2 mM K^+, 4.0 mM Ca^{2+}, 1.82 mM Mg^{2+}, 12.4 mM NO_3^-, 3.32 mM SO_4^{2-}, 10 mM P, 35 µM Fe^{3+}, 8.0 µM Mn^{2+}, 5.0 µM Zn^{2+}, 20 µM B, 0.5 µM Cu^{2+}, 0.5 µM MoO_4^{2-}). Plants were examined three times a week for stolons, flower bud appearance, anthesis, number of flowers and tuberization. A destructive harvest including dry weight measurements of tubers and shoot (aboveground stem, leaves and shoot apex) was done after eight weeks.

Experiment 3. Removing the tuberization signal: reducing StSP6A expression

Plants of *S. andigena* wild type and two *StSP6A RNAi* lines (#1 and #13) were transplanted to 17cm Ø pots and placed in a climate chamber (details can be found in the Appendix, Table S5.1). In addition to the three light treatments used in Exp. 2, a long-day treatment of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (LD400) was applied. Plants were grown and examined as in Exp. 2. Additionally, flower bud development was recorded (flower-bud size was categorized from zero to five where zero was no flower bud and five was an open flower). This was done due to the bad flowering success of *S. andigena* and the low chances of it reaching anthesis. The destructive harvest took place after eight weeks of growing and included fresh and dry weight measurements of tubers and shoot.

Experiment 4. Removing the tuberization signal and tuber sink: reducing StSP6A expression and removing stolons

S. andigena wild type and *SP6A RNAi* #13 plants were transplanted to 17cm Ø pots and placed in a short-day chamber with 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light from fluorescent tubes (Philips; Master TL-D Reflex 58W/840 Coolwhite) (details of the experiment can be found in the Appendix, Table S5.1). In half of the wild-type *S. andigena* plants, stolons were removed as soon as they formed. Stolons were also removed in half of the *SP6A RNAi* #13 plants to determine whether removing stolons affected plant growth in non-tuberizing plants. Plant growth control and determination of tuberization time and flower bud appearance were performed as in Exp. 2 and 3. Because flower bud size was only categorized and not measured precisely in Exp. 3, flower bud size was measured three times a week in Exp. 4. After appearance of the first flower bud, flower bud development was determined by measuring the diameter of the biggest flower bud on each plant.

Experiment 5. Increasing the tuberization signal: overexpressing StCDF1 in long days

An additional experiment was performed to confirm that StSP6A affected flower bud development. Instead of reducing *StSP6A* in short days, *StCDF1* overexpressing lines were used with upregulated *StSP6A* expression in long days. Plantlets of wild-type CE3027, and two lines overexpressing *StCDF1* (*35S::StCDF1#3* and *35S::StCDF1 #4*) were transferred to 15 cm Ø pots filled with a clay-peat mixture and placed in a long-day chamber with 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light from fluorescent tubes (Philips; Master TL-D Reflex 58W/840 Coolwhite) (details of the experiment can be found in the Appendix, Table S5.1). Plant growth control was performed as in Exp. 2, 3 and 4. Photographs of the shoot apex were taken after eight weeks of growing.

RNA analysis

StSP6A expression was analyzed to determine if the *StSP6A* silenced lines were indeed silenced in *StSP6A* and if the *StCDF1* overexpressing lines had upregulated *StSP6A.* Furthermore, *StSP3D* expression was analyzed to determine if the *StSP6A* silenced lines did not increase expression of the flowering signal *StSP3D*. Leaf samples of the plants in Exp. 3 were collected after five weeks, just before the first tuberization started. The fifth leaf from the top was sampled one

hour after the lights went on. Leaves from three plants were collected, pooled into one sample and frozen in liquid nitrogen and stored at -80ºC. Leaves were also collected from Exp. 5. The fourth and fifth leaf from the top were collected after five weeks, two hours after lights went on. Two plants were pooled and four pools per genotype were made. Gene expression was determined using qPCR (quantitative reverse transcription polymerase chain reaction). Frozen leaf material was ground and used for RNA extraction with an RNeasy plant mini-kit (Qiagen). A spectrophotometer (NanoDrop, Thermoscientific, Thermofisher) determined RNA concentration and quality. A DNase treatment was performed using Amplification grade DNase I (Invitrogen, Thermofisher). 1µg of RNA was used for cDNA synthesis with an iScript kit (Bio-Rad). RNA extraction, DNase treatment and cDNA synthesis were performed as described in the supplied manufacturers' protocols. 20 µl of cDNA was diluted to a total volume of 150 µl. 5 µl of SYBR-green (iQ-SYBR-green super mix, Bio-Rad), 0.25 µl Forward Primer (10 µM), 0.25 µl Reverse Primer (10 µM), 0.5 µl Milli-Q water and 4 µl diluted cDNA were used for the qPCR. In Exp. 3 three technical replicates were used per pooled sample. Samples were placed in a Thermal Cycler (C1000, Bio-Rad) set to 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by 95°C for 10 seconds and for a melt curve 65°C to 95°C in 0.5°C steps every 5 seconds. Primers used for *StSP6A* (PGSC0003DMT400060057) were (F) GACGATCTTCGCAACTTTTACA, (R) CCTCAAGTTAGGGTCGCTTG and for *StSP3D* (scaffold PGSC0003DMB00000014, unannotated) were (F) GGACCCAGATGCTC-CAAGTC, (R) CTTGCCAAAACTTGAACCTG and for *StNAC* (reference gene *NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX ALPHA*, PGSC0003DMT400072-220) were (F) ATATAGAGCTGGTGATGACT, (R) TCCATGATAGCAGAGACTA. Primers for *StSP6A* and *StSP3D* were used in (Navarro *et al.*, 2011) and the *StNAC* primer had an efficiency of 99%.

Data analysis

A student's t-test was used to compare two treatments and a one-way analysis of variance (ANOVA) was used to compare more than two treatments. A Bonferonni pair-wise comparison was used to determine which treatments significantly differed ($\alpha = 0.05$, IBM, SPSS Statistics 22 and GenStat, 18th Edition). When data was ordinal or not normally distributed (tested with a Shapiro-Wilk W-test for non-normality in GenStat), a non-parametric Kruskal-Wallis test and

Dunn's pairwise comparisons (α = 0.05) were computed in SPSS. Comparisons between light treatments in Exp. 2 and 3 were based on biological replicates as independent experimental units. For gene expression analysis three technical replicates were used for the qPCR analysis in Exp. 4 and four biological replicates were used for qPCR analysis in Exp. 5. $100/2^{-\Delta Ct}$ was used to determine gene expression values. Ct (cycle threshold) values of the gene of interest (*StSP6A* and *StSP3D*) were used to determine expression of the gene of interest compared to the housekeeping gene *StNAC*. Invariant expression of *StNAC* under the tested conditions is shown in the Appendix, Fig. S5.1.

Results

Removing the tuber sink: grafting onto a non-tuberizing rootstock

In order to establish how the absence or presence of tubers would affect flowering of the scions, two grafting experiments were performed in short-day and long-day conditions.

Grafting under short day conditions

Short-day conditions strongly promote tuberization. To determine whether flower bud development in CE3027 would improve without tubers, we grafted CE3027 scions onto non-tuberizing *S. etuberosum* and *S. palustre* rootstocks and grew them in short-day conditions. CE3027 scions underwent floral transition and as expected the flower buds failed to develop in the control grafts with tuberizing CE3027 rootstocks. Moreover, the buds also failed to develop when the CE3027 scion was grafted onto the non-tuberizing *S. etuberosum* or *S. palustre* rootstocks. Thus, the absence of tubers could not improve flower bud development. Opposite grafts were made with *S. etuberosum* and *S. palustre* scions on CE3027 rootstocks to determine how tubers would affect flower bud development. However, in the short-day conditions, neither *S. etuberosum* nor *S. palustre* transitioned to flowering and the CE3027 rootstocks failed to tuberize. To gain a better understanding on the effect of tubers on flower bud development, and attempt to induce flowering in *S. etuberosum,* a grafting experiment was performed under long-day conditions.

Grafting under long day conditions

Grafting CE3027 scions onto non-tuberizing *S. etuberosum* rootstocks did not improve flower bud development compared to the control grafts, which tuberized (CE3027 scion on CE3027 rootstock) (Fig. 5.2). Surprisingly, the opposite effect was observed. Although all tested graft combinations underwent flower transition (data not shown), grafts with CE3027 scions on *S. etuberosum* rootstocks only reached anthesis in 50% of the plants, whereas 82% of the control grafts reached anthesis (Fig. 5.3). Furthermore, the grafts with *S. etuberosum* rootstocks had almost half the number of open flowers compared to control grafts. The grafts made with scions of the early tuberizing genotype CE3130 also had impaired flower bud development in grafts with non-tuberizing rootstocks (Appendix, Table S5.2A).

To determine if the presence of tubers would impair *S. etuberosum* flower bud development, we made opposite grafts with *S. etuberosum* scions on

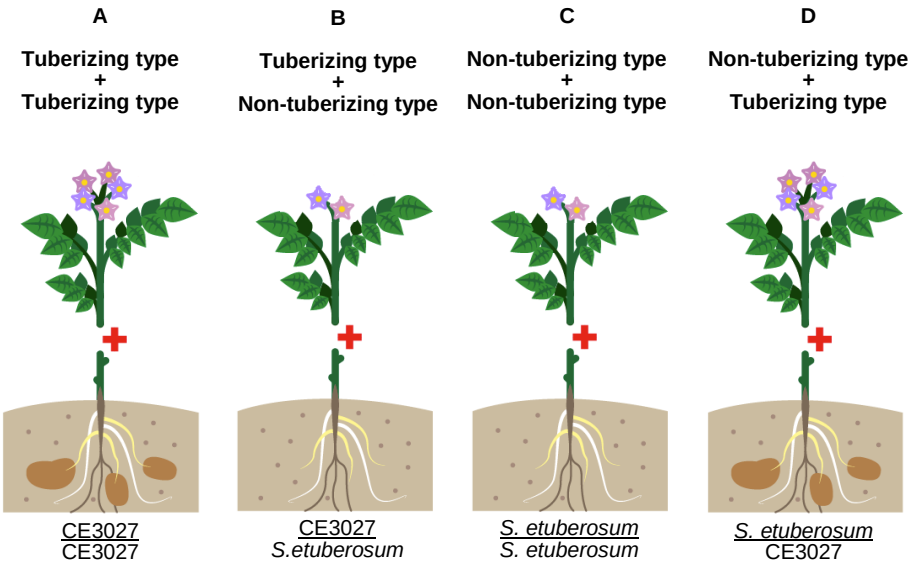


Figure 5.2. Schematic representation of flowering and tuberization in the grafting combinations between a tuberizing and non-tuberizing genotype, in long days. Potato genotype CE3027 is able to tuberize, while *S. etuberosum* is unable to tuberize. (A) The control grafts of CE3027 made tubers. (B) Grafts with CE3027 scions and *S. etuberosum* rootstocks did not make tubers. (C) The control graft of *S. etuberosum* did not make tubers. (D) Grafts with *S. etuberosum* scions and CE3027 rootstocks did make tubers, with or without leaves on the rootstock. All graft combinations formed buds which developed into open flowers. The graft combinations with a tuberizing rootstock (A, D) formed more open flowers than grafts without tuberizing rootstocks (B, C).

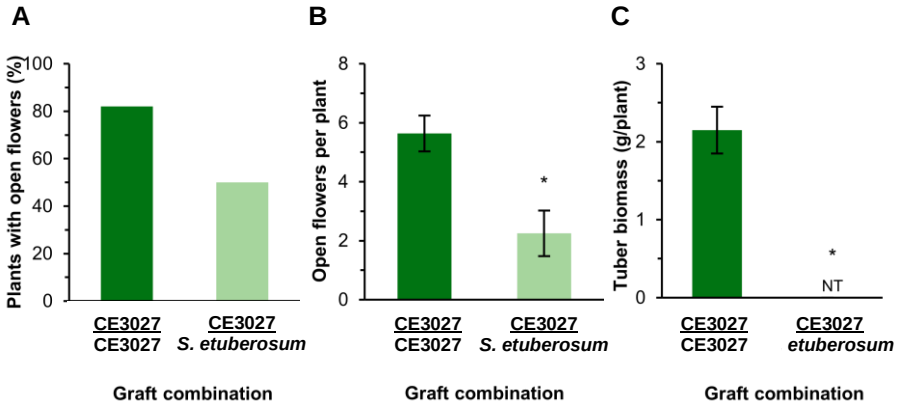


Figure 5.3. Flower bud development and tuber biomass in grafts with potato scions (CE3027) and tuberizing rootstocks (CE3027) or non-tuberizing rootstocks (*S. etuberosum*) in long days. (A) The percentage of grafts that reached anthesis (open flowering stage). (B) The maximum number of open flowers on a plant. (C) The dry weight of the tubers per plant at harvest, NT = no tuberization (biomass 0). The asterisk represents a significant difference between grafts with a tuberizing rootstock and a non-tuberizing rootstock, α = 0.005. Error bars show standard errors of the mean.

tuberizing CE3027 rootstocks. In contrast to the short-day grafting experiment, flower transition occurred in *S. etuberosum* and the flower buds developed into open flowers. Furthermore, CE3027 rootstocks tuberized, even when *S. etuberosum* scions were grafted onto them. However, a larger fraction of grafts with *S. etuberosum* scions on tuberizing CE3027 rootstocks reached anthesis, than of control grafts with *S. etuberosum* scions on non-tuberizing *S. etuberosum* rootstocks (Table 5.2). When comparing grafts in which the CE3027 rootstock was completely defoliated, with grafts in which some leaves were kept below the graft junction, the presence of leaves accelerated tuberization in CE3027

Table 5.2. Flower bud development and tuberization in grafts with *S. etuberosum* scions and *S. etuberosum* or CE3027 rootstocks (with or without leaves).

	Rootstock	Anthesis %*	Max. open flowers/plant	Tuber dry weight (g/plant)
Scion *S. etuberosum*	*S. etuberosum* (non-tuberizing)	33	3.33 a**	0.00 a
	CE3027 (tuberizing)	38	3.00 a	1.03 a
	CE3027 + leaves (tuberizing)	50	1.70 a	1.77 a

* The percentage of plants that reached the open flower stage

** Identical alphabetical letters indicate no significant difference between groups (α = 0.05)

rootstocks with approximately nine days (data not shown). Also, a larger fraction of grafts with leafy CE3027 rootstocks reached anthesis than grafts with leafless CE3027 rootstocks. Grafts with *S. etuberosum* scions on the early tuberizing CE3130 rootstocks showed a similar result. Anthesis was higher in grafts with CE3130 rootstocks and the presence of leaves accelerated tuberization, and also increased the number of plants with open flowers. (Appendix, Table S2B). Thus, tuberizing rootstocks did not impair the flower bud development of *S. etuberosum* scions.

Taken together, the interspecific grafting experiments did not show that the presence of tuber sinks impaired flower bud development, but rather had an unexpected opposite outcome where an improved flower bud development was observed in grafts producing tubers. To validate that these results were not due to interspecific interaction in the grafts, we performed another experiment where the tuber sink was removed within the same genotype.

Removing the tuber sink: removing stolons

To determine whether tubers negatively influenced flower bud development, tuberization was prevented by removing the stolons in CE3027 plants. Removing stolons did not significantly affect the percentage of flowering plants nor the number of open flowers per plant (Table 5.3). Also, the time until anthesis was not affected by removing the stolons (data not shown). The light conditions

Table 5.3. The effect of removing stolons on CE3027 flowering and plant biomass in different light treatments. Biological replicates, *n* = 11.

Light treatment	Stolons	Anthesis (%)*	Max. open flowers per plant	Tuber DW** (g/plant)	Shoot DW (g/plant)	Shoot + tuber DW (g/plant)
SD200* **	Intact	18	0.3c****	4.2b	2.2d	6.5b
	Removed	18	0.2c	0.0c	5.5b	5.5c
SD400	Intact	91	1.2bc	10.1a	2.4cd	12.5a
	Removed	91	2.4b	0.0c	8.0a	7.8b
LD200	Intact	100	6.5a	4.1b	3.5c	7.6b
	Removed	100	6.2a	0.0c	7.8d	7.8b

*The percentage of plants that reached the open flower stage

** DW = dry weight

*** Number indicates light intensity in $\mu mol \cdot m^{-2} \cdot s^{-1}$, SD = short day (8 hours), LD = long day (16 hours)

**** Identical alphabetical letters indicate no significant difference between groups ($\alpha = 0.05$)

under which plants were grown did affect flower bud development. The percentage of flowering plants was low in low-light short days (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$), but in high-light short days (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$) the percentage of flowering plants was almost as high as in long days (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Nevertheless, removing stolons did not improve flower bud development, both under short-day and long-day conditions. These results confirm that short-day conditions impair flower bud development in CE3027. Although the percentage of flowering plants was similar in a high-light short day compared to a low-light long day (SD400 and LD200 had the same DLI), the maximum number of open flowers per plant was significantly higher in the long day treatment (1.2 flowers in SD400 vs. 6.5 in LD200).

Tuberization took place in all light treatments unless stolons were removed (Table 5.3). The short-day treatment with high light intensity resulted in the fastest tuberization and the highest tuber biomass. Plants without stolons had a higher shoot biomass than plants with stolons. The light treatments with the highest DLI (SD400 and LD200) had a higher shoot biomass than the low light short day (SD200), in both tuberizing and non-tuberizing plants. The total biomass (tuber + shoot) was highest in the high light short-day treatment.

In summary, preventing tuberization by removing the stolons did not improve flower bud development, even though flower bud development was impaired in short days.

Removing the tuberization signal: reducing *StSP6A* expression

To determine whether the tuberization signal negatively influenced flower bud development, we used transgenic plants with reduced expression of the tuberization signal *StSP6A*. Flower buds were formed in all *S. andigena* plants, but flower bud development of the *S. andigena* wild type was impaired in short days compared to long days (Fig. 5.4). Wild-type plants under high light short-day and low light long-day conditions (SD400 and LD200) received the same DLI, but flower buds were smaller in the short-day treatment (Fig. 5.5A, $P = 0.02$). In the *StSP6A RNAi* lines grown under short-day conditions, flower bud development was improved compared to the wild type. Under these conditions, flower bud development in the *StSP6A RNAi* lines equaled the level of flower bud development in the wild-type plants under long-day conditions (Fig 5.5A, no significant difference between short day *StSP6A RNAi* lines and long day wild-

Figure 5.4. Flower buds in *S. andigena* in short and long days. Flower buds in high-light short days (SD400, 8/16 hours light/dark, 400 μmol·m^{-2}·s^{-1}) and low-light long days (LD200, 16/8 hours light/dark, 200 μmol·m^{-2}·s^{-1}), five weeks after transplanting and 8 weeks after transplanting.

type lines, P = 0.12). Moreover, two of the five *StSP6A RNAi* #13 plants in the high-light short days reached anthesis, which did not occur in *S. andigena* wild-type or *StSP6A RNAi* plants in any other light treatment, not even in long days (Fig. 5.5B). In long days, a lower *StSP6A* expression did not have an effect on flower bud development. Reducing *StSP6A* expression did not affect the flower initiation time in either short or long days. Gene expression analysis of *StSP6A* in *StSP6A RNAi* lines show that these lines were indeed silenced in *StSP6A* (Appendix, Fig. S5.2A). As expected, tuberization in the transgenic lines with reduced *StSP6A* expression, was inhibited compared to the wild-type plants (Appendix, Fig. S5.2B). Wild-type plants in high light long-day conditions showed a later and reduced tuberization compared to the plants in short days, while low light long-day plants did not tuberize at all. In summary, our results show that inhibiting tuberization by reducing *StSP6A* expression in potato plants grown under short-day conditions improves flower bud development.

Removing the tuberization signal and the tuber sink: reducing *StSP6A* expression and removing stolons

As the experiments testing the removal of the tuber sink used different genotypes than the experiments testing removal of the tuberization signal *StSP6A*, we performed a short-day experiment with *S. andigena*, where the tuberization signal *StSP6A* and the stolons were removed. Also, flower bud development was measured in more detail, to better illustrate differences between treatments. Plants with reduced levels of *StSP6A*, clearly developed

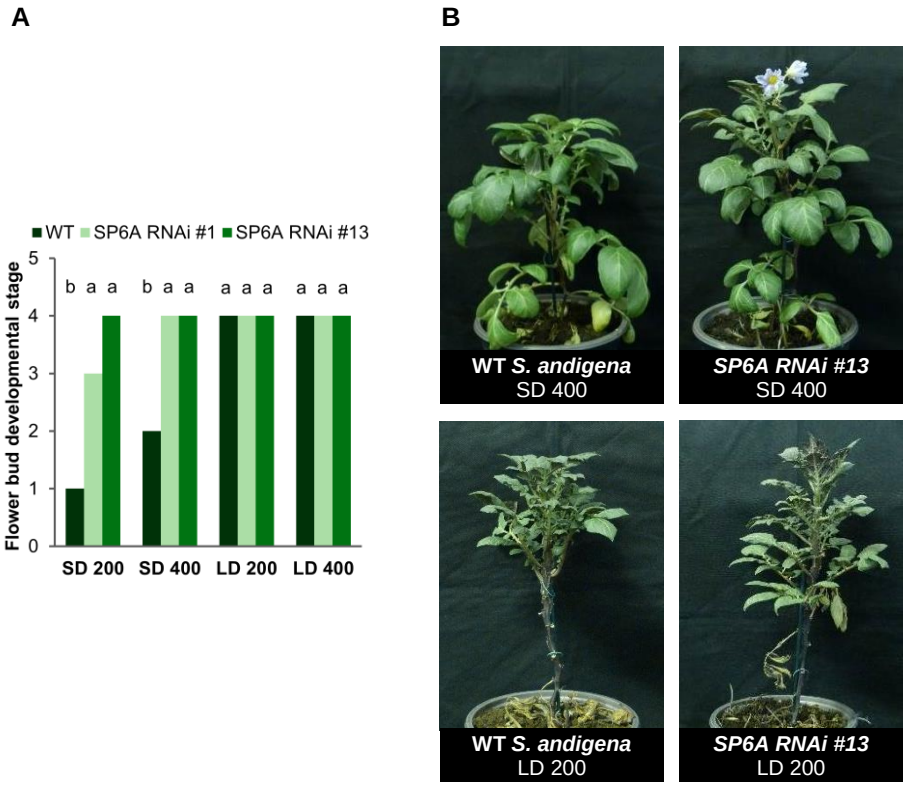


Figure 5.5. The effect of photoperiod and light intensity on flower bud development in *S. andigena* wild-type and *StSP6A RNAi* plants. Four light treatments were used: SD200 (short day, 8/16 hours light/dark, 200 µmol·m^{-2}·s^{-1}), SD400 (short day, 8/16 hours light/dark, 400 µmol·m^{-2}·s^{-1}), LD200 (long day, 16/8 hours light/dark, 200 µmol·m^{-2}·s^{-1}) and LD400 (long day, 16/8 hours light/dark, 400 µmol·m^{-2}·s^{-1}). SD400 and LD200 have the same daily light integral. (A) Flower bud development was categorized by size where 0 was no bud and 5 was an open flower. The median of the furthest stage of bud development during growing is given. Identical letters indicate no significant difference between genotypes in a light treatment ($\alpha = 0.05$). Biological replicates *S. andigena, n* = 8 and *StSP6A RNAi* lines, *n* = 5. (B) Plants at harvest: a wild-type *S. andigena* in SD400, a *StSP6A RNAi* #13 plant in SD400, a wild-type *S. andigena* in LD200 and a *StSP6A RNAi* #13 plant in LD200.

larger flower buds than wild-type plants, as in Exp. 3 (Fig. 5.6). The only plant to reach anthesis was a *StSP6A RNAi #13* plant without stolons. However, only removing the stolons did not significantly affect the flower bud size. Reducing *StSP6A* expression or removing the stolons did not affect the flower bud appearance time, which occurred on average after 28 days in all treatments (data not shown). The results show flower bud development is improved when the tuberization signal is removed, but not when only tubers are removed.

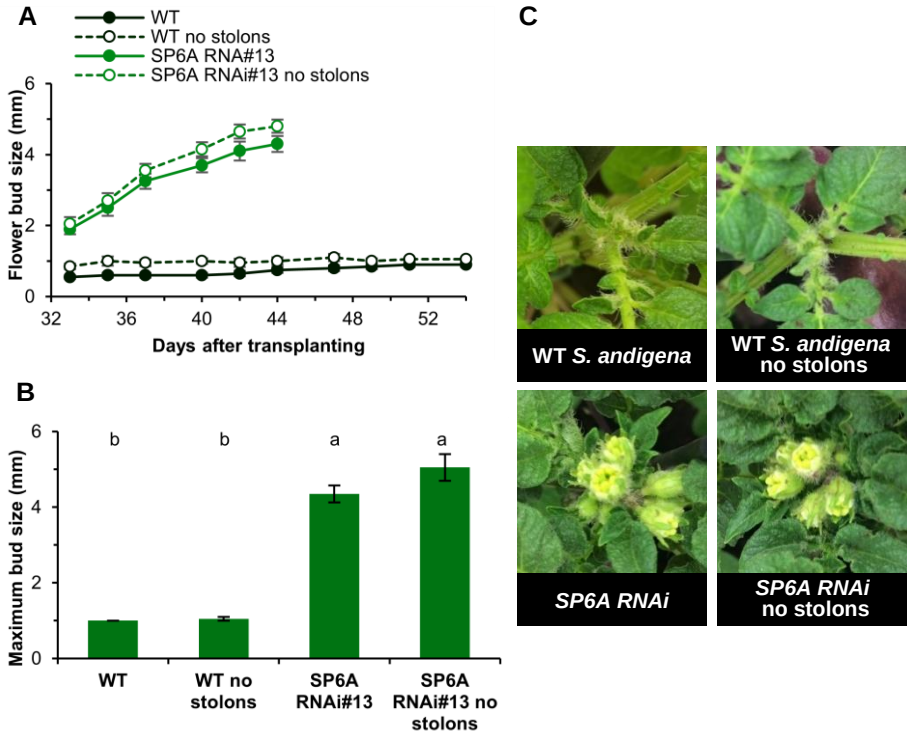


Figure 5.6. The flower bud development in *S. andigena* in a wild-type and a *StSP6A RNAi*#13 line where the stolons were either left intact or removed. Plants were grown in short days (8/16 hours light/dark) with a light intensity of 400 µmol·m^{-2}·s^{-1}. WT = wild type. (A) Flower bud size during growth (measurements were ceased when flower bud abortion started) and (B) maximum flower bud size reached by the plant. Error bars show standard error of the mean. Letters indicate significant differences in maximum flower bud size between treatments (α = 0.05). Biological replicates, *n* = 10. (C) Flower buds six weeks after transplanting in wild-type and *StSP6A RNAi#13 S. andigena*, with or without stolons.

Increasing the tuberization signal: overexpressing *StCDF1* in long days

StCDF1 overexpressing lines in a CE3027 background were used to confirm that *StSP6A* impairs flower bud development. Both *StCDF1* overexpressing lines in long days had upregulated *StSP6A* expression compared to the wild type (Fig. 5.7A). The flower bud development in these lines was inhibited and plants did not reach anthesis (Fig. 5.7B). The wild-type CE3027 plants were able to reach anthesis in long days.

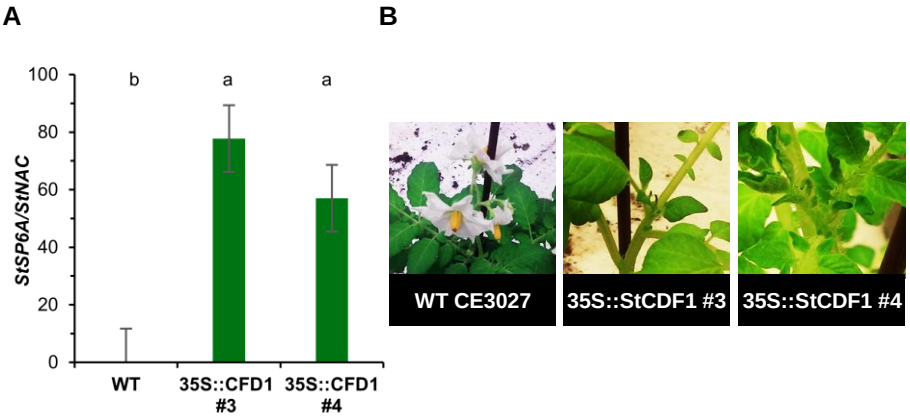


Figure 5.7. *StSP6A* expression and flowering phenotypes in wild-type CE3027, *35S:StCDF1* #3 and *35S:StCDF1* #4. Plants were grown in long days (16/8 hours light/dark) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. (A) *StSP6A* is expressed relative to the reference gene *StNAC*. Expression is from plants five weeks after transplanting. The error bars show the standard error of difference of the ANOVA analysis. Alphabetical letters indicate significant differences ($\alpha = 0.05$). Biological replicates, $n = 4$. (B) The shoot apex in wild-type and transgenic CE3027 plants eight weeks after transplanting. Genotypes from left to right: wild type, *35S:StCDF1* #3 and *35S:StCDF1* #4.

Plant growth after removing the tuber sink

In the experiments where tubers were removed, but the plants remained induced to tuberize, the plants showed abnormal growing patterns. In the grafting experiments in short days, scions of tuberizing genotypes on non-tuberizing rootstocks formed aberrant side shoots. Although these structures were green and lacked the characteristic hook found on stolon tips, they resembled stolons (Fig. 5.8A-B). These stolon-like structures were also found in long days, in grafts with scions of the early-tuberizing CE3130 on non-tuberizing rootstocks. Stolon-like structures were also formed on the stems of potato plants in inducing short days (Fig. 5.8C-D), where stolons were removed. These "aerial stolons" grew towards the soil and in some cases, as soon as the stolon reached the soil, tubers were formed at the tip (Fig. 5.8B). In other cases, these plants even started forming tubers directly on the stem (Fig. 5.8D). Potato plants that were induced to tuberize, but unable to do so in the conventional way, found alternative means of tuberization.

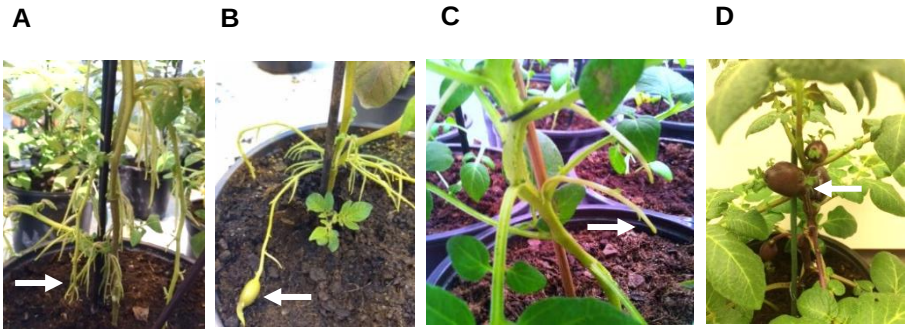


Figure 5.8. Stolon-like side-shoots formed under tuber inducing conditions if tuberization was impaired. (A) A graft where the scion of a plant that was able to tuberize was grafted onto a wild non-tuberizing rootstock (CE3027 / *S. palustre*) in short days. Stolon-like structures are formed above the graft unison (white arrow). (B) In the same graft combination the stolon-like structures on the scion formed a tuber upon reaching the soil. (C) In the CE3027 plants where the stolons were removed in short days, stolon-like structures were formed aboveground on the stem. (D) *S. andigena* plants grown in short days where the stolons were removed, formed tuber-like structures directly on the stem.

Discussion

Grafting with non-tuberizing rootstocks did not improve flower bud development

Long-day grafts with *S. etuberosum* rootstocks did not form tubers, but reached anthesis less often than the tuberizing control grafts and produced less open flowers when anthesis was reached (Fig. 5.3A-B). This is in line with results in opposite grafts, where the effect of tuberizing rootstocks on *S. etuberosum* scions was tested; in these grafts the fraction of plants with open flowers increased compared to control grafts with *S. etuberosum* rootstocks (Table 5.2). The results show that removing the tuber sink does not improve flower bud development.

That tuberizing rootstocks did not impair, but improved flower development in *S. etuberosum* scions, was surprising. Instead of inhibiting flower development, tuberization may improve flowering in a different species (*S. etuberosum*). The FT of one species can induce flowering or tuberization in another species, for instance with rice *Heading date 3a* (*Hd3a)* in potato, *Arabidopsis FT* in tomato and tobacco, and tomato *SINGLE FLOWER TRUSS (SFT)* in Arabidopsis (Lifschitz and Eshed, 2006; Lifschitz *et al*., 2006; Navarro *et al*., 2011). Potato StSP6A from the rootstock may improve *S. etuberosum* flowering

in the scion. Interestingly, long-day grafts between *S. etuberosum* scions and leafless CE3027 or CE3130 rootstocks flowered and tuberized, while short-day grafts between *S. etuberosum* scions and the leafless CE3027 and CE3130 rootstocks did not (data not shown). Perhaps in long days, a leaf-derived FT from *S. etuberosum* induces tuberization, while in short days this signal is not produced. Potato plants are thought to have an auto-regulatory *StSP6A* loop, where leaf-derived StSP6A leads to upregulation of *StSP6A* in the stolons, enhancing the level of StSP6A for tuberization (Navarro *et al.*, 2011). Potato scions expressing rice *Hd3a* but not *StSP6A,* have induced *StSP6A* in the stolons (Navarro *et al.*, 2011). If FT from *S. etuberosum* also induces this auto-regulatory loop, *S. etuberosum* FT may induce tuberization in the CE rootstocks and amplify the amount of FTs in the graft, possibly enhancing flowering as well.

Most importantly our grafting experiments show that the tuber sink does not impair flower bud development. However, because interspecific grafts were used, effects on flowering may have been caused by other properties of the *S. etuberosum* than its inability to tuberize. Therefore, to determine whether removal of the tuber sink improves flower bud development, stolons were removed in potato plants.

Removing stolons did not improve flower bud development

As with grafting, removing the stolons did not improve flower bud development in both CE3027 and *S. andigena* genotypes (Table 5.3 and Fig. 5.6). This is in line with previous experiments on stolon abscission (Weinheimer and Woodbury, 1966). Removing stolons also had no effect on flower initiation. The lack of stolons did lead to an increase of assimilates available for the shoot, as seen in the significant increase in shoot biomass (Table 5.3). However, this increase in shoot biomass did not improve flower bud development.

In short days, flower bud development was impaired compared to long days (Table 5.3). However, by raising short-day light intensity to match the DLI of long days, the percentage of plants that reached anthesis increased from 18% to 91%, which almost rivalled long-day anthesis (100%). Sufficient light is crucial for flower bud development, as has been demonstrated in several crops including potato and tomato (Kinet, 1977;Demagante and Zaag, 1988; Turner and Ewing, 1988). Increasing light may increase the amount of assimilates formed in the plant. Assimilates like sucrose play an important role in flower induction and floral development in potato (Chincinska *et al.*, 2008).

Nevertheless, the number of open flowers was significantly higher under long-day conditions, indicating an impairment of CE3027 flowering in short days, as was found before in other potato genotypes (Turner and Ewing, 1988). Thus, short-day flower bud development was impaired, and preventing formation of the tuber sink by removal of the stolons did not improve the flower bud development.

Removing the tuber sink in a plant that was induced to tuberize led to "aerial stolons"

Removing the tubers did not improve flower bud development. Although the plants were unable to tuberize, they were still induced to do so. Grafts that could not tuberize, started to produce stolons and tuber-like structures on the scions (Fig. 5.8A-B). Plants without stolons, growing in short days, also made stolon-like structures on the stem (Fig. 5.8C-D). Alternative tuberization structures have been documented before (Thijn, 1954; Weinheimer and Woodbury, 1966) in conditions where tuberization is prevented but plants remain induced to tuberize. The lack of tubers led to more assimilates in the shoot, but instead of promoting flowering these assimilates may have been directed towards alternative tuberization structures. The tuberization signal *StSP6A* is still expressed in inducing conditions, even when tubers are removed, which may be the cause of the direction of assimilates to alternative tuberization structures instead of to the flower buds. This theory is supported by the finding that the formation of stolon-like structures in short-day *StSP6A RNAi* plants was much less severe.

The tuberization signal StSP6A impairs flower bud development

S. andigena wild-type plants underwent floral transition in all tested light treatments, but in short days the flower buds ceased to develop at a very early stage (Fig. 5.5A). Remarkably, short-day flower bud development was significantly improved in the *StSP6A RNAi* lines. Two of the *StSP6A RNAi* plants were even able to reach anthesis in short days, which did not happen in any other treatment and is uncommon for *S. andigena* when grown in our climate chamber conditions. Flowering in *StSP6A RNAi* plants was also tested by Navarro *et al.* (2011), but only transition to flowering was considered and not flower bud development. The transition to flowering occurred at the same time as in the wild

type, as was the case in our experiments (data not shown). The transgenic lines had a significantly reduced *StSP6A* expression (Appendix, Fig. S5.2A). Improved flower bud development in the transgenic lines could not be explained by an increase in transcription of the proposed flowering signal *StSP3D* in the leaves (data not shown), implying that *StSP6A* negatively affects flower bud development through a different mechanism.

StSP3D has been proposed to be the flowering signal in potato, because silencing *StSP3D* showed a late flowering response (Navarro *et al.*, 2011). However, there is a lack of correlation between flower bud development and *StSP3D* expression, which is strongly expressed under short-day conditions but weakly expressed under long-day conditions (in prep. Dr. S. Bergonzi). Perhaps low expression levels of *StSP3D* are sufficient to induce flowering and the level of *StSP6A* determines the success of flower bud development. To fully understand potato flowering, elucidating the role of StSP6A in flower bud development, as well as StSP3D in flowering time and development, will be crucial.

Our finding that StSP6A represses flower bud development, while the tuber sink does not, was confirmed in another experiment testing both stolon abscission (tuber sink) and silencing of *StSP6A* (tuberization signal), in *S. andigena* in short days. Removal of stolons did not improve flower bud development, while downregulation of *StSP6A* did. The repressing role of StSP6A on flower bud development was further confirmed in CE3027 *StCDF1* overexpressing lines, with upregulated *StSP6A* in long days. The flower bud development was impaired in these lines and resembled the impaired flower bud development found in wild-type *S. andigena* plants in short days. Transgenic lines in which an upstream regulator of *StSP6A* was overexpressed were used instead of *StSP6A* overexpressing lines, to induce *StSP6A* in long days. In Navarro *et al.* (2011), transgenic lines overexpressing *StSP6A* actually improved flowering, perhaps by the strong and ubiquitous expression of *StSP6A* by the 35S promotor (Odell *et al.,* 1985; Seternes *et al.*, 2016). In the *StCDF1* overexpressing lines, the down-stream regulation on *StSP6A* is still intact, allowing a more realistic upregulation of *StSP6A* than in a *35S::StSP6A* overexpressing line. Flower impairment in these lines confirms our earlier findings that StSP6A represses flower bud development.

Can the inhibiting effect of a tuber sink be ruled out?

Although flower bud development was not improved by tuber-sink removal in CE3027, CE3130 or *S. andigena*, removing the tuber sink had a positive effect on flowering in some genotypes in the past (Thijn, 1954; Jessup, 1958). However, these reports have also been contradicted (Turner and Ewing, 1988). Therefore, it may be possible that repression of flower development by the tuber sink is genotype specific. It would be interesting to find out if reducing *StSP6A* would further improve flower development in genotypes that are benefitted by tuber-sink removal. Nevertheless, our findings show that in *S. andigena* and CE3027 the tuber sink does not repress flower bud development while the tuberization signal StSP6A does.

The day-length control of flowering in potato

Short days, or more correctly long nights, induce tuberization in potatoes, although variation exists between varieties in their dependence on short days (Garner and Allard, 1923; Ewing and Struik, 1992; Prat, 2010; Kloosterman *et al.*, 2013). Potato flowering has been categorized as a short-day, long-day and day-neutral process (Jones and Borthwick, 1938; Turner and Ewing, 1988; Almekinders and Struik, 1994; Martínez-García *et al.*, 2002; Schittenhelm *et al.*, 2004). A cause for this large variation might be the use of different genotypes and the difference in definition of flowering. Because flowering is a process composed of many phases, it needs a clear distinction when addressed: it starts with floral initiation and proceeds with flower bud and organ development. Our results show that flower bud appearance occurs independently of the photoperiod and tuberization, but that flower bud development is repressed by the tuberization signal. Anthesis was only attained in short days with high irradiance in plants where *StSP6A* was repressed (*StSP6A RNAi#13*), indicating that photoperiod and irradiance also play a role in the development of flower buds. Our results point to a short-day control of flower bud development in potato, but due to internal control by StSP6A, flower bud development is promoted under long-day conditions.

Interaction between two modes of reproduction in potato

A likely mode of action for StSP6A to impair flower bud development, could be through control of assimilates. Although removing the tuber sink did not improve flower bud development, it cannot be claimed assimilates do not play a role, as alternative tuber structures that acted as sinks were still formed unless *StSP6A* was silenced. StSP6A may have a role in directing assimilates towards tuberization, which consequentially could be detrimental for flower development, especially if tuberization takes place while flower buds are still developing. How this direction of assimilates takes place remains to be elucidated. Whether flowering is actually repressed by StSP6A may be genotype specific and depend on the timing of both tuberization and flowering. The European Cultivated Potato Database (https://www.europotato.org) shows a huge variation in flowering success between varieties and it has been suggested that potato berry and seed development is impeded by earliness of tuberization (Pallais, 1987). Similar findings were seen in the CE3027 and CE3130 control grafts, where the early tuberizing CE3130 grafts flowered less profusely than the later tuberizing CE3027 grafts (Fig. 5.3B and Appendix, Table S5.2). It would be interesting to correlate the tuberization time and *StSP6A* expression to the flowering time and flower developmental success in a large number of genotypes.

While two reproduction modes may inhibit each other in the same species, interspecies interaction between reproduction modes may be beneficial for both processes, as was seen in *S. etuberosum* scions grafted on CE3027 and CE3130 rootstocks. The flowering in *S. etuberosum* scions was improved compared to control grafts with *S. etuberosum* rootstocks. The *StSP6A* may not function as an inhibiter in *S. etuberosum* because flowering and tuberization are not competing processes in this species. Consequently, StSP6A may substitute FT in *S. etuberosum* and improve flowering, while StSP6A inhibits flowering in potato.

Conclusion

Our results show that flower bud development in potato is impaired by the tuberization signal StSP6A, and not by the tuber sink itself. These results suggest there is an internal mechanism in potato plants where one mode of reproduction can affect the other.

Acknowledgements

We thank the Centre of Genetic Resources (CGN) seedbank for providing *Solanum etuberosum* and *Solanum palustre* seeds. Special thanks to Priscila Malcolm Matamoros for help with the grafting and Simonetti and Beekenkamp Plants for providing grafting clips. This research is part of the FlowerPot project which was supported by the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO), Signify, Solynta, Beekenkamp Plants and Incotec. We thank Signify for providing the LEDs for this project.

Appendix

Table S5.1. Overview of the five experiments testing how tuberization affects potato flower bud development.

Exp.	Method of tuber removal	Plants per treatment	Genotypes	**Conditions:** day length (hours), day/night temperature (°C), light intensity (µmol·s^{-1}·m^{-2}), relative humidity, duration (weeks), location
1a	**Removing tuber-sink**: Grafting	10	CE3027 *S. etuberosum* *S. palustre*	8, 22/18**, not measured (winter-spring 2016), not measured, 14, greenhouse
1b	**Removing tuber-sink**: Grafting	10*	CE3027 CE3130 *S. etuberosum*	16-17, 22/18**, not measured (summer 2016), not measured, 12, greenhouse
2	**Removing tuber-sink**: Removing stolons	11	CE3027	8 & 16, 20/20, 200 and 400, 70%, 8, climate chamber
3	**Removing tuber signal**: Silencing *StSP6A*	8 5 5	*S. andigena* WT *StSP6A RNAi#1* *StSP6A RNAi#13*	8 & 16, 22/18, 200 and 400, 70%, 8, climate chamber
4	**Removing tuber signal and sink:** Silencing *StSP6A* and removing stolons	10 10	*S. andigena* *StSP6A RNAi#13*	8, 22/18, 400, 70%, 8, climate chamber
5	**Increasing the tuber signal:** Overexpressing *StCDF1*	10 8 8	CE3027 *35S:StCDF1 #3* *35S:StCDF1 #4*	16, 20/18, 200, 70%, 6, climate chamber

* Some grafting combinations exceeded or failed to reach this number (see Table 5.1)
** In the greenhouse in the daytime, temperatures sometimes exceeded the set temperature of 22ºC.

Table S5.2. Flower bud development and tuberization in grafts between CE3130 and *S. etuberosum*.

A	Rootstock	Anthesis %*	Maximum open flowers/plant	Tuber dry weight (g/plant)
Scion CE3130	CE3130 (tuberizing)	90	2.6 a**	8.0 a
	S. etuberosum (non-tuberizing)	10	0.4 b	0.0 b

B	Rootstock	Anthesis %	Maximum open flowers/plant	Tuber dry weight (g/plant)
Scion *S. etuberosum*	*S. etuberosum* (non-tuberizing)	33	3.3 a	0.0 b
	CE3130 (tuberizing)	36	3.0 a	2.3 b
	CE3130 + leaves (tuberizing)	42	3.8 a	6.5 a

* The percentage of plants that reached the open flower stage

**Identical alphabetical letters indicate no significant difference between groups (α = 0.05)

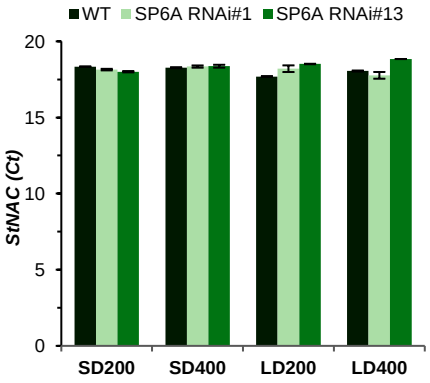


Figure S5.1. *StNAC* expression (Ct = cycle threshold) in wild-type *S. andigena, StSP6A RNAi* #1, and *StSP6A RNAi* #13. The reference gene *StNAC* is similarly expressed in all tested light treatments and genotypes. Plants were grown in short days (SD) of 200 and 400 µmol·m^{-2}·s^{-1} and long days (LD) of 200 and 400 µmol·m^{-2}·s^{-1}. The error bars show the standard error of the mean. Technical repetitions, *n* = 3.

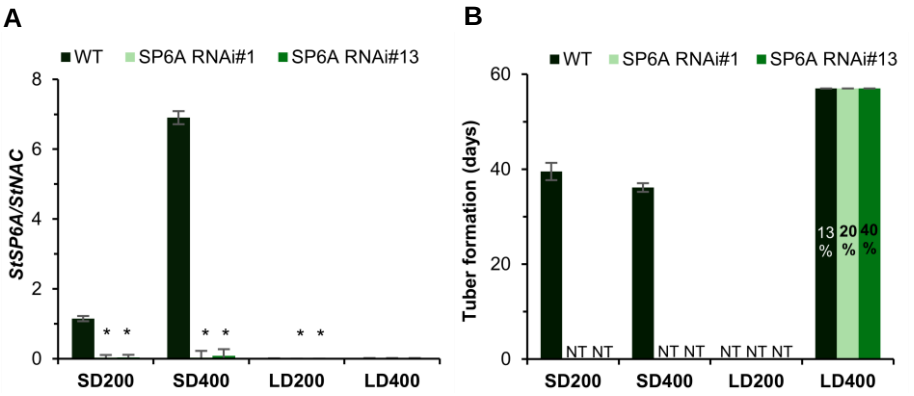


Figure S5.2. *StSP6A* expression and tuberization time in wild-type *S. andigena*, *StSP6A RNAi* #1, and *StSP6A RNAi* #13. Plants were grown in short days (SD) of 200 and 400 µmol·m^{-2}·s^{-1} and long days (LD) of 200 and 400 µmol·m^{-2}·s^{-1}. (A) *StSP6A* is expressed relative to the reference gene *StNAC*. Expression is from plants five weeks after transplanting. The error bars show the standard error of difference of the ANOVA analysis between genotypes. Asterisks indicate a significant difference to the wild-type expression in a given light treatment ($\alpha = 0.05$). Technical repetitions, $n = 3$. (B) Tuberization time in days after transplanting. The error bars show the standard error of the mean. No significant differences were calculated in SD200, SD400 and LD200because tuberization did not occur in the transgenic lines. No significant differences were found in LD400. The percentage of tuberizing plants is indicated in the bar (no indication means 100% tuberization). Biological replicates: Wild type ($n = 8$), *StSP6A RNAi* #1 ($n = 5$), *StSP6A RNAi* #13 ($n = 5$).

6. General discussion

Scope

Hybrid breeding in potato

Potato is one of the most important food crops in the world (International Potato Center, 2018). Studying potato development gives important insights that may improve potato production in the future. Furthermore, potato is an interesting crop because it can reproduce both asexually, through the formation of tubers, and sexually, through the formation of flowers, berries and seeds. In the past, tuberization has gained more attention than flowering, because tubers are the edible part of the plant and because tuberization is the main mode for propagation. New developments in potato breeding will shift some of the emphasis from tuberization to flowering. Hybrid breeding is an exciting new possibility in potato and although potato flowering has always been of interest to potato breeders, a new possibility to produce potato seeds for propagation will require increasing efficiency of the flowering process. This increased requirement necessitates an improved understanding of potato flowering, which has hardly been scientifically analyzed in the past. Of course, tuberization is still of great importance, as the final product is the tuber. Therefore, to optimize potato production using hybrids, both potato flowering and tuberization need to be understood.

Studying tuberization and flowering simultaneously

Understanding how both reproduction processes are regulated could enable breeders or growers to separately control both processes when required. Considering both tuberization and flowering are reproduction strategies, and specific conditions are required for optimal reproduction, it is plausible that both processes are similarly regulated by the environment. This thesis discusses for the first time, both tuberization and flowering simultaneously, while looking at different aspects of light. The goal was to understand how light regulates tuberization and flowering in potato.

I determined how (LED) light, which can be divided into spectrum, photoperiod, intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$) and daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$), regulates tuberization and flowering. Information on the molecular control behind tuberization was used to test a practical application of light spectrum on tuberization and potato flowering (Chapter 2). Furthermore, photoperiodic

control of tuberization was explored, by testing whether the well-studied coincidence model in Arabidopsis, between CONSTANS (CO) and light, also explained night-break inhibited potato tuberization (Chapter 3). Subsequently, the effect of the DLI on flowering was determined (Chapter 4), and in the final research chapter (Chapter 5), it was clarified whether competition takes place between flowering and tuberization and an explanation behind this competition was proposed.

In this General discussion I highlight the most important results in respect to the light spectral, photoperiodic and DLI-mediated effects on tuberization and flowering. The molecular control behind these processes is discussed and knowledge gaps and potential explanations are addressed. I propose a working model explaining the mechanism behind tuberization, flower initiation, flower development and competition between these processes. Finally, I conclude this chapter with the practical significance of these findings and determine if light can be used to create an optimal environment for potato flowering or tuberization.

Light as a regulator

In Fig. 1.1 (Chapter 1) a model was proposed, summarizing light regulated potato reproduction. This model was based on information from literature. Light was separated into three components: light spectrum, photoperiod and DLI (or alternatively light intensity). These components regulate tuberization and flowering through two main factors: potato CONSTANS (StCOL1) and assimilates. StCOL1 can act through the tuberization signal StSP6A and possibly the flowering signal StSP3D (Navarro *et al.*, 2011). Although it is known that assimilates are involved in tuberization and flowering regulation in potato (Xu *et al.*, 1998; Chincinska *et al.*, 2008; Navarro *et al.*, 2015), it was not yet clear if this regulation was mediated through *StSP6A* and *StSP3D*.

Major findings

Fig. 6.1. of this General discussion depicts a similar model, which illustrates the main effects of light on potato tuberization and flowering, observed in this thesis. Fig. 6.2 summarizes the main mechanisms underlying these light effects. Mechanisms and light effects will be discussed in the following sections.

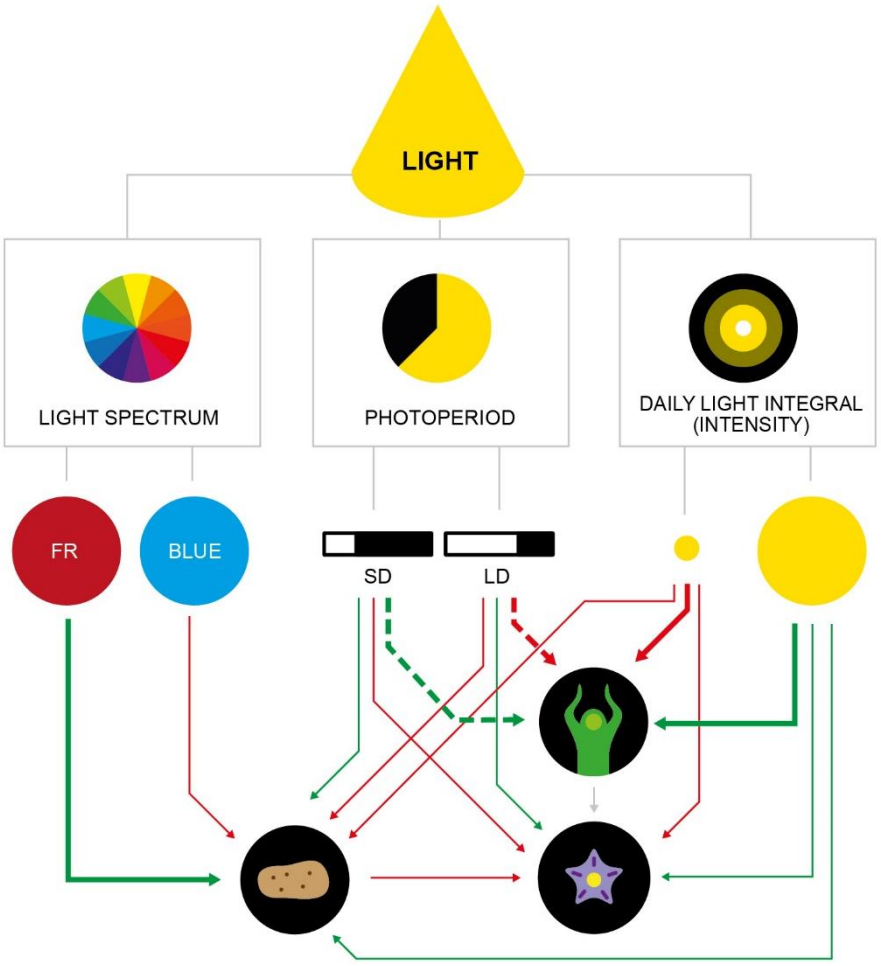


Figure 6.1. Light effects on tuberization and flowering in potato. All effects depicted with red and green arrows were demonstrated in this thesis. Bold arrows show effects that were not yet demonstrated before this thesis. Light acts through light color (far red and blue), short or long days (SD and LD) and through a low or high light level (small or large yellow circle), which is expected to act through the daily light integral, but alternatively may act through light intensity. Light acts on tuberization, floral induction (shoot apical meristem) or flower development (open flower)

Light spectrum

In this thesis it is demonstrated that far-red light can be applied to potato plants to accelerate tuberization (Chapter 2). This regulation is expected to be controlled through the reduced red to far-red ratio, which leads to less active phytochrome B (PHYB) (Sager *et al.*, 1988; Smith and Whitelam, 1997), reducing

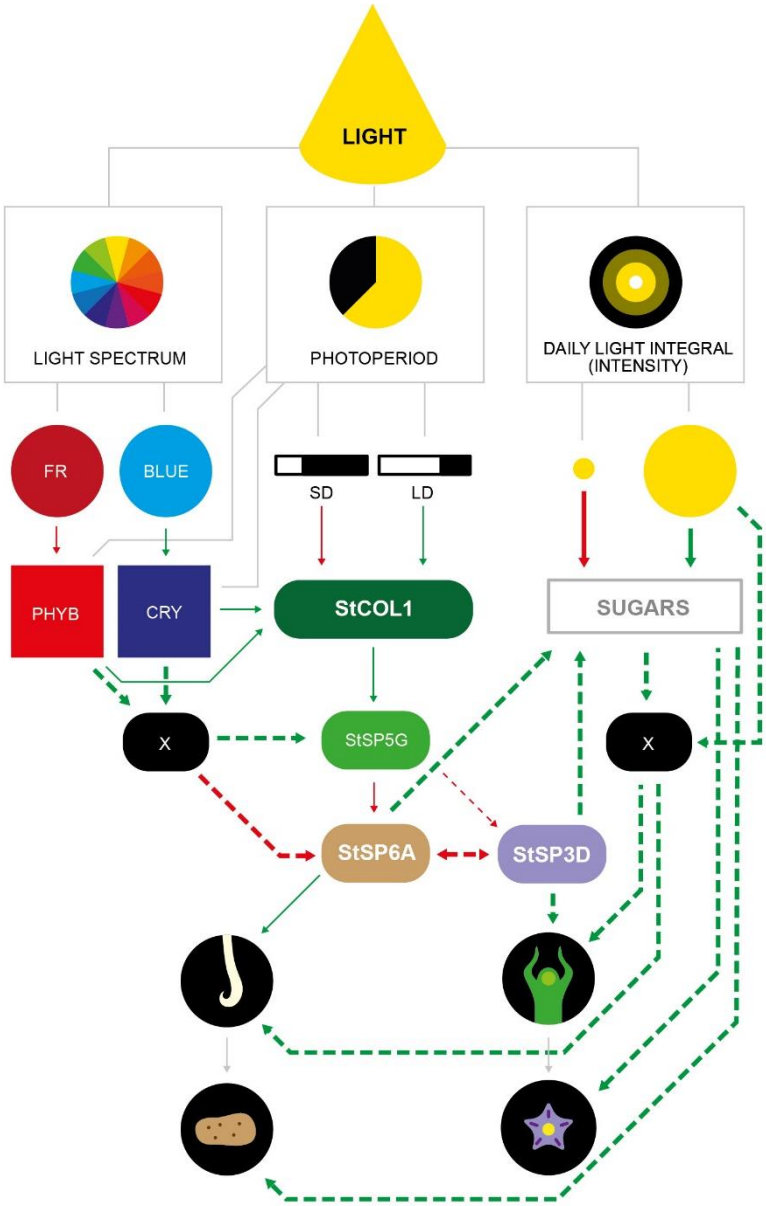


Figure 6.2. The regulation behind light-mediated potato reproduction. Bold arrows show effects that were not yet demonstrated before this thesis. Dashed lines indicate a possible regulation, which still has to be proven. Light acts through color (far red and blue), short or long days (SD and LD) and through low or high light levels yellow circles). Light level is proposed to act through the daily light integral, but may act through light intensity. The separate light factors act on photoreceptors (PHYB and CRY), CONSTANS (StCOL1) or plant sugars. In turn, potato *FTs StSP5G, StSP6A* and *StSP3D* are induced, and floral or tuber induction occurs (stolon and shoot apical meristem). Finally, assimilates (perhaps directed by StSP6A and StSP3D) allow further development (into potatoes and open flowers). "x" indicates unknown pathways .

the stabilization of StCOL1 (Abelenda *et al.*, 2016), which in turn leads to an upregulation of *StSP6A* (Navarro *et al.*, 2011). Also, the effect of light spectrum on potato flowering is demonstrated for the first time. Flowering time was not affected by the far-red treatments, suggesting StCOL1 does not control *StSP3D* or alternatively, StSP3D does not determine flowering time. Blue light delayed tuberization time and this delay may be controlled through the photoreceptor cryptochrome (CRY). Blue light activates CRY (Cashmore *et al.*, 1999) and CRY, like PHYB, is expected to stabilize StCOL1 (Abelenda *et al.*, 2016). Thus, blue light may delay tuberization through extra stabilization of StCOL1. However, an alternative mode of control is more likely, as will be explained later on. Interestingly, when the day length was extended with blue light, the inhibiting effect on tuberization was less severe than when days were extended with white light. A possible explanation for this finding is discussed below. As was the case for far-red light, blue light did not affect flowering time, leading to the assumption that light spectrum does not control flowering time in potato, although tuberization is clearly affected.

Photoperiod

Photoperiodically regulated tuberization is in part controlled through the light spectrum. Coincidence of light and the circadian clock regulated CO is known to control flowering in Arabidopsis (reviewed in: Andrés and Coupland, 2012). Light leads to activation of photoreceptors that stabilize CO, which in turn induces *FT,* the flowering time gene. In potato, photoperiodic regulation has also been proposed to act through coincidence of light and *StCOL1* expression (Abelenda *et al.*, 2016). Stabilization of StCOL1, through active PHYB and CRY, plays an important role in this regulation. In this thesis it is shown that coincidence between light and *StCOL1,* and stabilization of StCOL1, does not necessarily lead to inhibition of tuberization. A night break applied at the end of the night when *StCOL1* expression was at its peak, was not able to induce *StSP6A* and tuberization. Contrarily, a night break applied in the middle of the night, with low *StCOL1* expression, led to repressed *StSP6A* and inhibited tuberization (Chapter 3). These findings suggest there is an additional light-mediated control affecting tuberization, independently of StCOL1 (Fig. 6.2). I propose that the suggested mechanism behind photoperiodic tuberization, where *StCOL1* peak expression coincides with light and upregulates *StSP5G,* downregulates *StSP6A* and inhibits tuberization, is not complete. Additional regulation is necessary to

explain the results acquired in Chapter 3. Interestingly, although flowering time in days was not affected by photoperiod, the number of leaves formed before the inflorescence was decreased in short photoperiods, indicating flower initiation was accelerated (Chapter 3). This finding demonstrates that flowering may be under photoperiodic control, and thus also under light spectral control, in contrast to what was found in Chapter 2. However, only the leaf number was altered by photoperiod, not the time till flower bud appearance.

Daily light integral and light intensity

Although the DLI and light intensity are connected, I propose the effect of high light on potato flowering to be controlled through the DLI (Chapter 4). Furthermore, this effect is expected to be controlled by another mechanism than photoperiod and light spectrum (Fig. 6.2). This thesis demonstrates for the first time that an increase in DLI accelerates flower initiation in potato (Chapter 4). The results in this thesis indicate that accelerated flowering under high DLIs is not regulated through upregulation of the flowering time gene *StSP3D*, but is mediated through an unknown pathway, perhaps involving assimilates (Fig. 6.2). Furthermore, the DLI was shown to improve flower bud development. Additional results not shown in Chapter 4, also demonstrate that high DLI accelerates tuberization (Appendix, Exp. S1). A positive effect of the DLI on tuberization was demonstrated before (Demagante and Van der Zaag, 1988). However, based on findings described in this General discussion, I propose that this acceleration is not mediated through *StSP6A* (Appendix, Exp. S1, further discussed below).

Competition between tuberization and flower bud development

A final addition to the suggested model in Fig. 6.2 is competition between StSP6A and StSP3D. Competition between tuberization and flowering has been suggested to act through the sink strength of the developing tubers (Almekinders and Struik, 1996). However, the final research chapter in this thesis (Chapter 5) shows that the tuberization signal StSP6A represses flower bud development. Removing the tuber sink in short-day conditions did not improve flower bud development, while repressing *StSP6A* did. Inducing *StSP6A* in long-day conditions led to a similar repression of flower bud development as was seen in short-day conditions. In Chapter 5 it was suggested that the repressive role of

StSP6A acts through its function in directing assimilate distribution in the plant. A working model explaining this theory, will be further discussed below.

Pathways behind the regulation of potato reproduction by light

As introduced above, I present a new model for the control behind the regulation of tuberization and flowering by light (Fig. 2). Although many processes are still uncertain, the results in this thesis give an indication of the complexity behind this control. In the previous chapters a large part of the regulation of tuberization and flowering was discussed in regard to the well-studied photoperiod pathway, and the less studied carbohydrate pathway. However, another well-known pathway regulating potato reproduction that was not studied in this thesis, is the plant hormonal pathway. Plant hormones like gibberellic acid (GA), jasmonates and cytokinins play important roles in regulating reproduction in potato. Additionally, the environment has been demonstrated to have a clear effect on plant hormone levels (Prat, 2010). However, this control is complex. For example, it is generally accepted that GAs inhibit tuberization, but there is a nuance to this control where not only the form of GA is crucial for this effect, but also the localization of the hormone (Prat, 2010). Determining the involvement of several plant hormones, in several forms and located in various plant tissues, was too ambitious for this project. However, more research on the role of plant hormones in the regulation of potato flowering and tuberization will be crucial to fully understand potato reproduction in response to light.

Nevertheless, in this General discussion, the focus will be on light regulated potato reproduction through StCOL1 (photoperiod and light spectrum) and through assimilates (DLI and competition).

Regulation of potato reproduction through potato CONSTANS

Photoperiodic regulation

Photoperiodic flower initiation

Flower bud appearance time was not affected by the photoperiod. However, the number of leaves formed before the inflorescence was smaller in short days,

demonstrating an acceleration in flower initiation in shorter photoperiods. Thus, flowering may be under the same control as tuberization. However, decreased leaf number in short days was only found in *S. andigena* (Chapter 2, Table 2.2 and Chapter 4, Fig. 4.1B, D), indicating photoperiodic flowering control may be genotype specific. This genotype specificity fits with past documentation of flower initiation in potato, where flowering was shown to be a short-day or day-neutral processes, depending on the study and on the genotype used (Firman *et al.*, 1991; Almekinders and Struik, 1994; Almekinders and Struik 1996). In our study, the genotype with the same leaf number in short and long days was less day-length sensitive than *S. andigena*, as it was able to tuberize in long days. However, in this genotype, short days did have an accelerating effect on tuberization, which suggests the photoperiodic control on tuberization is stronger than on flowering time. It must be noted that although inhibited flowering in *S. andigena* in short-day conditions is shown in Chapter 2, this result is most likely not caused by short-day inhibition of flower initiation. Presumably, the flower bud appearance was not perceived due to the very small size of the flower buds in short day conditions. In Chapters 3, 4 and 5, where flower bud appearance time was observed more precisely, both long and short days led to flower initiation in this genotype.

How can flower initiation be regulated by photoperiodically controlled StSP3D?
The reduced number of leaves found in *S. andigena* under short-day conditions indicates that flower initiation may be under photoperiodic control. The proposed flowering signal StSP3D is also under photoperiodic control, as it is highly expressed in short days and very lowly expressed in long days. Nevertheless, the flower initiation does not correspond to *StSP3D* expression, as flower initiation occurs after only a few more leaves in long days, while *StSP3D* expression is very low in these conditions. Also, the flower initiation (number of leaves) in the genotype CE3027 was not affected by photoperiod, while *StSP3D* expression was very lowly expressed in long days (Chapter 4, Fig. 4.4). Furthermore, the flower bud appearance in both CE3027 and *S. andigena* occurred at the same time under short- and long-day conditions. Low expression of the tuberization signal *StSP6A* in long days leads to inhibition of tuberization, while for flower initiation the level of *StSP3D* expression seems less important, how come? It may be the case that potato flowering is not controlled by StSP3D,

however, past studies demonstrate that silencing *StSP3D* represses flowering (Navarro *et al.,* 2011). Therefore, another explanation must be sought.

Several possibilities were discussed in Chapter 4, including the presence of more *FTs* controlling flowering, like in Chrysanthemum (Sun *et al.*, 2017), or the fact that very low levels of StSP3D may be sufficient to induce the very sensitive shoot apical meristem (Abelenda *et al.,* 2014). Chapter 4 also discusses a model where the ratio of repressor and inducer of flowering is important for flowering control. A similar model was shown in Soyk *et al.* (2017), where accelerated tomato flowering could be attained even when *SFT* was partially knocked down, as long as the floral repressor *SP5G* was inhibited. A model explaining potato flowering through the interplay of *StSP3D* and an inhibiting *FT*-homolog *StSP5G,* was rejected in Chapter 4 (Discussion). However, the ratio between *StSP6A* and *StSP3D* may be more important in this regulation. In short days, both *StSP6A* and *StSP3D* are highly expressed, but *StSP6A* has the highest expression (Navarro *et al.*, 2011). While in long days, both *FTs* are lowly expressed. If StSP6A inhibits StSP3D, very little StSP3D would be available in both short and long days. If the shoot apical meristem is indeed sensitive to low amounts of StSP3D, repression by StSP6A may explain how floral transition can occur in both photoperiods. However, Chapter 5 shows that silencing *StSP6A* does not accelerate the flowering time, and thus the ratio between StSP6A and StSP3D may not be relevant for floral initiation (but may be more important for flower development as will be discussed below).

A final explanation for the contradicting *StSP3D* expression and flower bud appearance time was also shortly discussed in Chapter 4. StSP3D may not be a mobile signal like StSP6A (Navarro *et al*., 2011), but may be locally expressed in the shoot apical meristem, as is the case for the flower inducer *AcFT2* in onion (Lee *et al.,* 2013). In this case, the *StSP3D* expressed in the leaves, as measured in Chapter 4, would not be relevant for flower initiation. As the shoot apical meristem is less exposed to the environment than the leaves, it may be the case that flower initiation is not controlled by the environment, but by internal cues. *StSP3D* expression profiles indeed indicate *StSP3D* is expressed in long and short days in shoot apical meristematic tissue (Bergonzi *et al.*, in prep.). If *StSP3D* expression in the shoot apical meristem is the cause for flower initiation, very low levels of StSP3D may be sufficient to induce flowering, because the signal is very localized. It has often seemed puzzling that *StSP3D* silenced lines were able to delay flowering time (Navarro *et al.*, 2011), while the low *StSP3D* expression

measured in the leaves in long days could not (Chapter 4). *StSP3D* expression in the shoot apical meristem may explain this discrepancy. Quantifying *StSP3D* expression in the shoot apical meristem in wild-type and *StSP3D* silenced lines, in long and short days, could determine if there is a correlation between *StSP3D* expression and flower initiation.

Present understanding of photoperiodic control on tuberization

A coincidence model has been proposed for photoperiodic control of tuberization, which is similar to a coincidence model in Arabidopsis (Navarro *et al.,* 2011; Andrés and Coupland, 2012; Abelenda *et al.,* 2016). In this potato model, first a set of circadian controlled genes (*StGI* and *StFKF1*) determine how *StCOL1* is expressed throughout the day. By forming a complex with each other in long days, the circadian clock genes can degrade StCDF1, which is a repressor of *StCOL1* (Navarro *et al.*, 2011; Kloosterman *et al.*, 2013). Coincidence of *StCOL1* expression with light allows for StCOL1 to be stabilized, which leads to induction of *StSP5G* and in turn, repression of *StP6A* and tuberization (Abelenda *et al.*, 2016). However, in potato, *StCOL1* is expressed in both long and short days, but in short days, *StCOL1* expression peaks at the end of the dark period (Chapter 3, Fig. 3.3A), while in long days, *StCOL1* expression peaks at the beginning of the light period (Chapter 3, Fig. 3.3M). Thus it was previously proposed that the peak expression of *StCOL1* must coincide with light to exert its inducing function on *StSP5G,* and repress *StSP6A* and inhibit tuberization (Abelenda *et al.*, 2016). A simplified model of photoperiodic regulation of tuberization is shown in Fig. 6.3.

In this thesis, tuberization control is largely explained through the photoperiodic pathway and the tuberization signal StSP6A. However, the BEL1-like transcription factor, StBEL5, is also thought to be involved in tuberization control in potato (Chen *et al.* 2003). StBEL5 has been reported to induce *StSP6A* in the leaves and additionally the *StBEL5* RNA transcript is transported to the stolons where it may induce tuberization locally (reviewed in: Hannapel *et al.*, 2017). Although not studied in this thesis, it would be very valuable to determine how *StBEL5* is affected under different light conditions, to discover if light regulated tuberization could be controlled through StBEL5, next to the proposed control through StSP6A, summarized in Fig. 6.3.

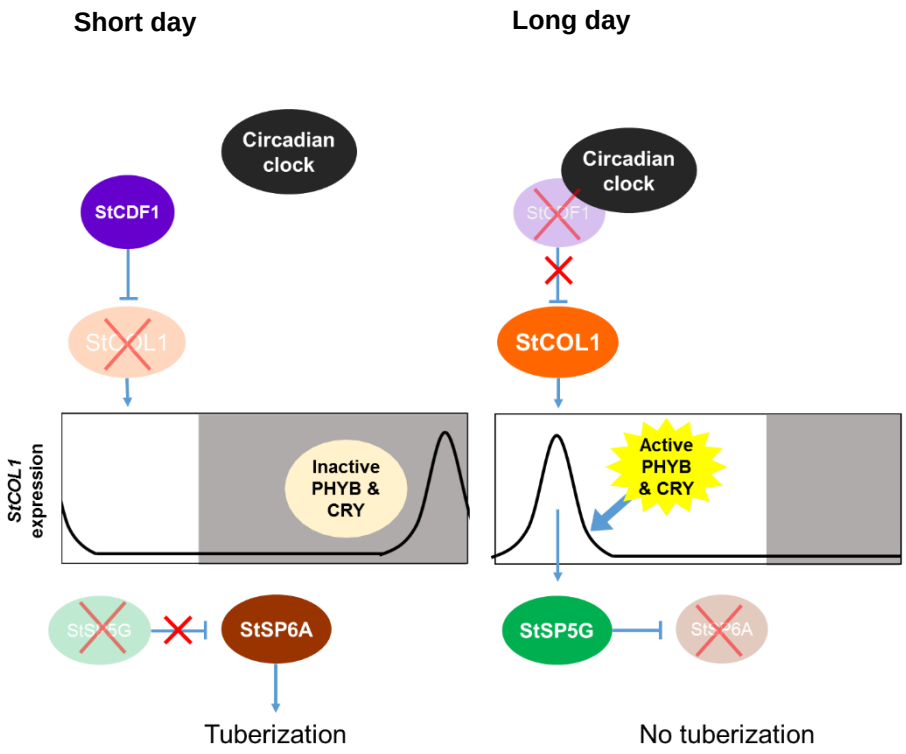


Figure 6.3. Photoperiodic control on potato tuberization based on a coincidence model. In short days a repressor or *StCOL1*, StCDF1, represses *StCOL1* and *StCOL1* expression peaks in the dark period, not allowing for StCOL1 stabilization and repression of *StSP6A*, thus leading to formation of StSP6A and induction of tuberization. Due to control by circadian clock genes in long days, StCDF1 is degraded and the *StCOL1* expression peak is shifted into the light period at the beginning of the day. This leads to stabilization of StCOL1 and the induction of *StSP5G*, which represses *StSP6A* expression, and inhibits formation of the tuberization signal StSP6A and thus tuberization.

Timing light with StCOL1 expression to regulate tuberization

This thesis demonstrates with the use of night breaks, that coincidence of light and peak *StCOL1* expression does not always inhibit tuberization, which contradicts the present hypothesis. Although a night break given in the middle of the night inhibited tuberization in *S. andigena*, the night break in the middle of the night was applied before *StCOL1* peak expression, which occurred at the end of the dark period. At the time of peak *StCOL1* expression, western blot analyses showed that the StCOL1 protein was degraded, thus the night break in the middle of the night most likely did not lead to much stable StCOL1. Surprisingly, the

night break at the end of the dark period, which coincided with peak *StCOL1* expression and was shown to lead to stabilized StCOL1 at this time, did not inhibit tuberization. Moreover, a night break applied at the beginning of the dark period, which did not coincide with *StCOL1* expression in the night, delayed tuberization. These puzzling results cannot support the coincidence model as a way of explaining day-length dependent tuberization. As shown in Chapter 3, a straight-lined control through StCDF1, StCOL1, StSP5G, and StSP6A cannot always explain tuberization. Based on these findings I suggest there are more complex connections between these factors and perhaps several unknown genes involved in this pathway. As discussed in Chapter 3, an additional level of control may be regulated through the length of the night period, as has been proposed in other plants (Higuchi *et al.*, 2013).

An unknown factor dependent on night length may be involved in photoperiodic tuberization

StSP5G is upregulated even when *StCOL1* expression does not coincide with light in the early night-break treatment (NB12, Chapter 3, Fig. 3.3). Western blot analysis does not show stable StCOL1 in the dark period, at the time when *StCOL1* expression is upregulated. The question remains, what is inducing *StSP5G* if not StCOL1? It is possible that the upregulated *StCOL1* in the beginning of the light period is enough to induce *StSP5G*, as *StCOL1* expression was relatively high at ZT2 compared to the other short-day treatments (Chapter 3, Fig. 3.3). Alternatively, the early night break may have induced or stabilized a different factor which has a repressing effect on tuberization, perhaps through induction of *StSP5G*. The night break at the middle of the night does not coincide with peak *StCOL1* expression, but *StSP5G* is induced, suggesting the night break may coincide with peak expression of this unknown factor in the middle of the night. The late night break does coincide with peak *StCOL1* expression, but does not induce *StSP5G,* thus perhaps the late night break does not coincide with the unknown factor and this factor is crucial for successful repression of tuberization. Pearce *et al.* (2017) suggest that in the long-day plant wheat, effectiveness of a night break in inhibiting flowering is gated by one or more unknown circadian clock genes. In potato, next to the repressing role of StCOL1, an additional repressor of tuberization may be regulated in the dark period. Higuchi *et al.* (2013) suggested a similar model for night-break inhibited flowering in Chrysanthemum. The authors proposed a photosensitive period in

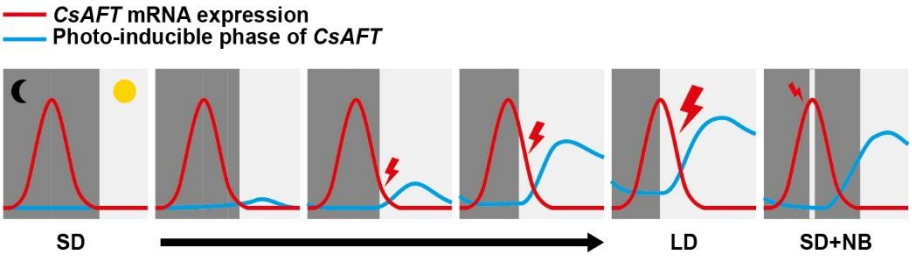


Figure 6.4. A model proposing the regulation of a floral repressor. This model shows the light inducible phase of a flowering repressor CsAFT in Chrysanthemum. This photo-inducible phase is indicated by the red line. The gate for maximal induction of *CsAFT* opens at a constant period after the dark period starts. This model indicates how *CsAFT* (black line) is induced in response to natural day-length extension and artificial lighting with night breaks. Lengthening of the light period (and shortening of the dark period), allows the light sensitive phase to coincide with light (lightning bolt), which leads to induction of *CsAFT* and the inhibition of flowering. Applying a night break (light period in the night in the last figure) allows the light of the night break to coincide with the photosensitive phase, which induces the floral repressor and inhibits flowering. This figure is adapted from Higuchi *et al.* (2013).

the night, where coincidence with light activates an inhibitor of flowering. The gate for maximal induction of the floral inhibitor opens at a constant time after the start of the dark period, regardless of the length of the light period. In a long day, light in the morning coincides with the light inducible phase of the floral repressor leading to inhibited flowering. When a night break is applied in the middle of the night, this also leads to coincidence of light and the light inducible phase of the floral repressor, which also inhibits flowering (Fig. 6.4, adapted from Higuchi *et al.* 2013). A logical parallel with this control in tuberization could be *StCOL1* expression in the night. However, *StCOL1* expression cannot be the likely candidate for this gated control, as coincidence between light and StCOL1 alone does not explain the results of the night-break treatments in Chapter 3. Also, *StCOL1* expression does not always take place at a fixed amount of hours after start of the dark period (Chapter 3, Fig. 3.3). However, a different, still unknown factor could be controlled through a similar mechanism, where the tuber repressing phase peaks in the middle of the night, instead of at the end like *StCOL1* expression.

A proposed model for the dark-period regulated tuberization repression

A similar model based on this unknown factor may explain the unexpected tuberization phenotypes in the night-break experiments in Chapter 3. As discussed in Chapter 3, light activated PHYB can remain active in the dark for a period of time before dark reversion inactivates it (Ruddat *et al.*, 1997;

Fankhauser, 2001). Night-break activated PHYB may remain active for some time, even after the night break is applied. Results from Chapter 3 show that dark reversion happens relatively quickly. Within two hours after applying the night break, StCOL1 is almost totally degraded (Chapter 3, Fig. 3.4), which may indicate there is not enough active PHYB to stabilize StCOL1. These results correspond with StCOL1 detection in Abelenda *et al.* (2016), in a line overexpressing *StCOL1*, which shows some StCOL1 after lights have been off for an hour but no StCOL1 after lights have been off for three hours. If the unknown tuber repressing factor is indeed stabilized by PHYB, the small prolonged action of this photoreceptor after a night break, may result in enough unknown repressing factor to be stabilized to inhibit tuberization (Fig. 6.5). In an early night break, the slow deactivation of PHYB may still allow some stabilization of the tuber repressing factor in the beginning of the night, while the late night break may only catch the end of the expression of the unknown repressor. Although PHYB is suggested here as an inducing/stabilizing factor, exclusive action through PHYB cannot be assumed based on the experiments in this thesis. Night breaks were applied with a white/red mix, thus other photoreceptors may also play a role.

The unknown factor may act on *StSP5G* expression. This idea is supported by the recent finding that the delaying effect of night breaks on tomato flowering is regulated through PHYB-mediated induction of *SP5G* (Cao *et al.*, 2018), which is a homolog of potato *StSP5G* (Soyk *et al.*, 2017). Because potatoes and tomatoes belong to the same family (Solanaceae), it is plausible that some control mechanisms remain conserved between the two species. PHYB activated in a night break, may stabilize the unknown factor which can induce *StSP5G,* leading to delayed tuberization. However, *StSP5G* expression is similar in the early and the regular night-break treatment, but the regular night-break treatment is expected to lead to more unknown tuber repressing factor (Fig. 6.5).

Instead of *StSP5G,* this unknown factor may repress *StSP6A* through another route. *StSP6A* expression and the tuberization phenotype correspond to the hypothetical stabilization/activation of the unknown repressing factor described in Fig. 6.5. Total repression of *StSP6A* and tuberization was found in long days and in the treatment with a night break in the middle of the night, but only partial repression of *StSP6A* was found in the early and late night-break treatments. In this case, it is surprising that the early night-break treatment had a strong repressing effect on tuberization, while the *StSP6A* expression was only slightly lower than in the late night-break treatment, where tuberization was not

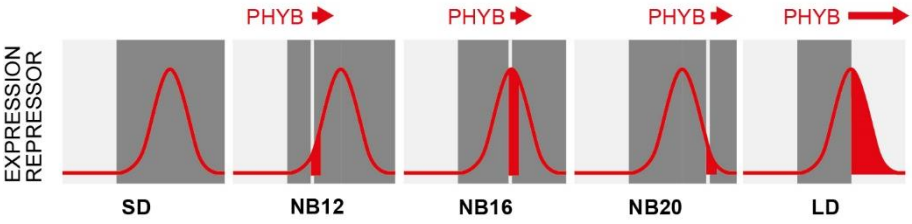


Figure 6.5. Hypothetical model showing the activation of a proposed tuberization repressor by light. When light coincides with the expression of an unknown factor (red line), a tuber repressing signal is produced/stabilized (red surface). Phytochrome B (PHYB) is activated by the light of a night break, which may stabilize/activate this unknown factor. Due to slow dark reversion, PHYB may remain active shortly after the night break is over, allowing for extra stabilization/activation of the unknown tuber repressor. In a short day (SD), light does not coincide with expression of the unknown repressor and tuberization can occur. In the early night break (NB12, applied 12 hours after the start of the light period), the slow inactivation of PHYB allows for more repressing factor to be made, compared to a late night break (NB20), because the PHYB remains active once expression of the unknown factor increases. A night break at the middle of the dark period (NB16) is even more repressing for tuberization, because the light coincides with maximum expression of the unknown factor. A long day (LD) would lead to the most repressing factor and inhibited tuberization, due to extended PHYB activity in the light period.

inhibited or delayed. Perhaps the small difference in *StSP6A* expression caused this difference in tuberization, or perhaps additional control downstream of *StSP6A* plays a role. More experiments are needed to determine which is the case.

Finally, it was discussed in Chapter 3 why plants overexpressing *StCOL1* had upregulated *StSP5G* in long and in short days, but still had slightly upregulated *StSP6A* in short days. Perhaps this effect is caused by the unknown factor, which is hypothesized to represses *StSP6A* in long days, and not in short days (Fig. 6.5). This would lead to partial repression of *StSP6A,* through StCOL1 in short days, but full repression of *StSP6A,* through StCOL1 and the unknown repressor in long days. Although results of the night-break experiments indicate that stable StCOL1 does not always lead to repressed tuberization, partial regulation of *StSP6A* through StCOL1 cannot be ruled out. It has been demonstrated that *StCOL1 RNAi* lines with repressed *StCOL1* expression had upregulated *StSP6A* in long days (Abelenda *et al.,* 2016), indicating the inhibiting effect of StCOL1 on *StSP6A* and tuberization.

Could tuberization also be controlled by StCOL2?

If tuberization control by photoperiod is indeed regulated through an additional unknown tuber repressing factor, what could this unknown factor be? A possible candidate that fits the expected expression pattern hypothesized in Fig. 6.5 is

StCOL2, a homolog of *StCOL1* (Abelenda *et al.*, 2016). Three tandemly arranged homologs of *CONSTANS* were identified in potato (*StCOL1-StCOL3*) and it was shown that *StCOL1* is expressed at relatively high levels in leaves, but *StCOL2* levels are much lower and *StCOL3* is almost undetectable (Abelenda *et al.*, 2016). Expression of *StCOL2* was shown to peak in the middle of a long night and the similarity to *StCOL1* may indicate it functions similarly in repressing tuberization. However, it was shown that StCOL2, in contrast to StCOL1, is stabilized by far-red light (Abelenda *et al.*, 2016). Although not tested in this thesis, previous studies have shown reversibility of a night break with far-red light (Batutis and Ewing, 1982), making StCOL2 an unlikely candidate controlling night-break induced inhibition of tuberization. Unless the far-red reversal acted on StCOL1, which only relieved some of the inhibition on tuberization, considering far red was not able to fully reverse the night-break inhibition (Batutis and Ewing, 1982).

Next to the possibility for additional control of *StCOL* homologs on tuberization, there may be a functional divergence in the different StCOL homologs in potato, where StCOL1 regulates tuberization through *StSP6A* and another StCOL regulates potato flowering through *StSP3D,* or another route. Additional study is needed to test this hypothesis.

Missing links in photoperiodic control of potato reproduction

StSP3D leaf expression does not correlate to the flower initiation time, but flower initiation may depend on shoot apical meristematic *StSP3D* expression instead, which may not be under a strong photoperiodic control. Determining if shoot apical *StSP3D* correlates with flower initiation will increase the general understanding of the photoperiodic regulation on flowering time.

Tuberization is under strong photoperiodic control and repression of tuberization in long days may only in part be controlled by StCOL1. An additional control, regulated by the duration of the dark period, may explain results where coincidence of light and peak *StCOL1* expression is not able to inhibit tuberization. The existence and identity of this unknown factor must still be determined to confirm this idea. Furthermore, it still needs to be elucidated how *StSP5G* is induced, as upregulation of this gene does not solely depend on StCOL1 presence. A combination of StCOL1 and the proposed unknown factor may regulate *StSP5G* expression. All in all, photoperiodic experiments show that the coincidence of *StCOL1* expression and light alone cannot explain the

photoperiodic regulation on tuberization. Additional control on *StSP5G* and on *StSP6A*, possibly mediated by a light stabilized unknown factor induced by the dark period, may fine-tune the tuberization process in varying photoperiods.

Light spectral regulation

Blue light repression of tuberization

As demonstrated by Abelenda *et al.* (2016) applying white light is more effective in stabilizing StCOL1 than applying red or blue light, which may mean both PHYB and CRY are involved in stabilization of StCOL1. By applying both blue and white light, as was done in the blue-light treatment in Chapter 2 (Table 2.1, W+B), both CRY and PHYB would have been active to stabilize StCOL1 and repress tuberization, which could explain the delay in tuberization seen in the RH genotype (Chapter 2, Fig. 2.2A). Even though blue light is known to have a weak deactivating role on PHYB (Sager *et al.*, 1988), the PHYB activating role of the red light in the treatment is much stronger. This is shown by the only slightly lower phytochrome stationary state (PSS) measured in the blue light treatment, indicating the activity of the phytochrome (1 is fully active and 0 is fully inactive) (Chapter 2, Table 2.1). However, two things do not add up when considering a repressing effect on tuberization. First, although StCOL1 is stabilized by the CRY that is activated by blue light, this does not explain why tuberization would be repressed compared to the control. In the white/red light of the control treatment, both PHYB (activated by the red LEDs) and CRY (activated by the blue light in the white LEDs) should already be activated. Thus, adding blue light would not have a big effect. Second, in short days, *StCOL1* expression primarily takes place in the dark period (Chapter 3, Fig. 3.3A) and therefore additional blue light in the light period is not likely to have an effect through StCOL1 stabilization. It could be possible that the effect of the photoreceptors is delayed and StCOL1 is stabilized in the dark period. Photoreceptors may act on other factors, which in turn could stabilize StCOL1 once it is expressed in the dark. However, my and previous western blot data indicate that activated photoreceptors in the light are not able to stabilize StCOL1 in the dark period (Chapter 3, Fig. 3.4, and Abelenda *et al.*, 2016). The idea that blue light does not repress tuberization through StCOL1 stabilization is supported by results from an experiment by Fixen *et al.* (2012). In this experiment continuous blue light led to inhibition of tuberization in one of the tested genotypes, while continuous red

light did not. As demonstrated in Abelenda *et al.* (2016), red and blue light are just as effective in stabilizing StCOL1. Thus if StCOL1 stabilization would be the only reason blue light represses tuberization, continuous red light should have repressed tuberization as well (as would continuous white light which was not tested). Thus, I propose that not StCOL1, but another factor underlies blue-light mediated tuberization repression.

CRY may have an additional effect on tuberization, which is not regulated through StCOL1. In Arabidopsis, CRY has a role in flower induction through StCOL1 stabilization, but can also induce *FT* independently of StCOL1 (Liu *et al.*, 2008; Pin and Nilsson, 2012). In the short-day potato, CRY may inhibit tuberization downstream of *StCOL1*, for instance by inducing *StSP5G*, which also is a homolog of *FT*, like *StSP6A* and *StSP3D* (Potato Genome Sequencing Consortium, 2011). Blue light may also inhibit tuberization by acting upstream of *StCOL1.* In Arabidopsis, blue light is required for the formation of the GI-FKF1 complex that degrades CDF and enables *CO* expression (Sawa *et al.*, 2007). If StGI and StFKF1 also require blue light to degrade StCDF1 in potato, blue light applied in short days may stimulate *StCOL1* expression and repress tuberization. Determining if the *StCOL1* expression is altered in the blue light treatment could confirm or rule out this possibility. Alternatively, Fixen *et al.* (2012) proposed that blue light exerts its effect through GA, which has been shown to inhibit tuberization, as mentioned previously (Jackson and Prat, 1996). Although a blue-light repression of tuberization may be mediated by GAs or by the above mentioned alternatives, the blue-light effect was genotype specific in both my and in Fixen *et al.'s* experiments. Genotype specificity makes it difficult to propose a mechanism of action with the molecular control that is now known. This molecular control is largely based on *S. andigena,* in which tuberization was not repressed by blue light (Chapter 2, Fig. 2.2A).

Far-red light acceleration of tuberization

In long days, *StCOL1* peaks in the morning. Applying far-red light throughout the light period should lead to StCOL1 degradation by deactivation of PHYB, which in turn should induce tuberization (Jackson *et al.*, 1996). Genotypes already able to tuberize in long days had accelerated tuberization in the far-red treatments. As these genotypes are able to tuberize in long days, it is very probable they have a truncated StCDF1 allele, which leads to a stronger repression of *StCOL1* (Kloosterman *et al.*, 2013). Reduced *StCOL1* expression, is expected to lead to less

repression of tuberization, enabling these genotypes to tuberize in long days. By adding far-red light, the tuberization time in these genotypes was accelerated to match tuberization time in short days (Chapter 2, Fig. 2.1A and Fig. 2.2A), probably by degrading the relatively small amount of StCOL1 that was still present. However, far red could not induce tuberization in the obligate short-day *S. andigena* (Chapter 2, Fig. 2.1A). Apparently, far-red light application did not lead to degradation of the relatively large amount of StCOL1 that was present in long days in *S. andigena*. Contrarily, *S. andigena* lines silenced in *PHYB* were able to tuberize in long days (Jackson *et al.*, 1996). Perhaps PHYB activity in the far-red treatments in Chapter 2 was not low enough, and some active PHYB was able to stabilize StCOL1. Indeed, PSS values in the far-red treatments only reached a minimum of 0.75 when applied throughout the day (Chapter 2, Table 2.1), even though 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of far red was added in the high far-red treatment. It is difficult to obtain very low PSS values, without harming the plant; low PSS values lead to excessive stretching of the stem (Chapter 2, Table 2.2). Therefore it would be more efficient to only apply a high dose of far-red light where it has the most effect. Timing far-red application is discussed in the following section.

Timing light spectrum

By combining both photoperiodic and light spectral regulation on potato reproduction, the understanding of both processes can be fine-tuned.

End-of-day far-red treatment acts on regulation in the dark

Low intensity end-of-day (EOD) far-red light was just as effective in accelerating tuberization as a high intensity far-red treatment during the whole light period. However, the timing of the EOD far-red light makes it unlikely that this treatment would function through a similar mechanism as far-red light given throughout the day. When far red is applied during the light period of a long day, it can accelerate tuberization by deactivating PHYB and destabilizing StCOL1, which is expressed in the light period. In contrast, an EOD far-red treatment is applied just before the dark period, when *StCOL1* is not expressed. The EOD far-red treatment functions by rapidly deactivating PHYB (Casal, 2013). When far red is not applied at the end of the day, PHYB is still deactivated through dark reversion, but this is a much slower process (Ruddat *et al.*, 1997; Casal, 2013). Therefore, the effectivity of an EOD far-red treatment may lie in the earlier inactivity of PHYB at the beginning of the dark period. Thus, the EOD far-red

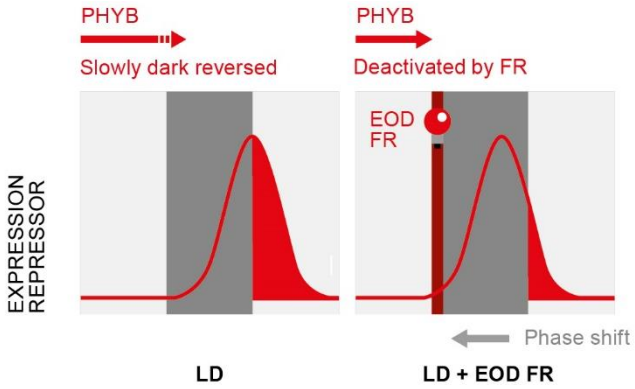


Figure 6.6. Hypothetical model showing the photosensitive phase of an unknown tuber repressor in response to an end-of-day (EOD) far-red (FR) treatment. When light coincides with expression of the repressor, the repressor can be stabilized and tuberization is repressed. The expression of this repressor depends on the duration of the dark period, which may be controlled through phytochrome B (PHYB). Once PHYB is inactive the expression may be induced (red line). At the end of a long day (LD, left pane) PHYB is slowly inactivated by dark reversion and it may take a while before the tuber repressor is induced (left pane). By applying an EOD far-red treatment (LD + EOD FR, right pane), PHYB is directly inactivated and the expression of the tuber repressor can start immediately. The shift in the expression phase causes for less coincidence with this phase and light in the morning and thus less repression of tuberization.

treatment has only a small window to be effective. Without far red, PHYB may have been dark reversed after approximately two hours (as discussed previously), therefore these two hours may be crucial to activate a process which affects tuberization. It is possible that the EOD far-red light causes the dark period to be sensed two hours earlier, perhaps shifting the expression phase of the unknown tuber repressor back into the dark period, and reducing the repressive effect on tuberization (Fig. 6.6).

Alternatively, PHYB may even affect *StCOL1* expression. The EOD far-red treatment may cause for the night to be sensed as a longer night, leading to a *StCOL1* expression which corresponds to a longer night, and thus a shorter day. In short days, *StCOL1* expression peaks a few hours earlier than in long days, just before the light period starts (Chapter 3, Fig. 3.3A, M). Thus, perhaps the fast PHYB inactivation of the EOD far-red treatment leads to a slightly earlier *StCOL1* peak, and partial expression in the dark period. Accordingly, there would be less coincidence with *StCOL1* expression and light at the beginning of the day and less repression of tuberization. Interestingly, *StCOL1* expression in *S. andigena* plants that are silenced in *StPHYB,* indeed peak somewhat earlier than wild-type plants (Abelenda *et al.,* 2016). Measuring *StCOL1* expression in plants receiving EOD far-red light could determine if tuberization was accelerated by a phase shift in

StCOL1 expression or by an alternative route, perhaps involving the unknown repressing factor.

Far red applied during peak StCOL1 expression in the morning of a long day
An additional experiment was performed that was not incorporated in the research chapters of this thesis (Appendix, Exp. S2). I performed this experiment to determine whether degrading StCOL1 with far-red light during peak *StCOL1* expression, would be able to induce tuberization in long days in *S. andigena*. The addition of far red during peak *StCOL1* expression in the light period of a long day did not induce tuberization in *S. andigena*. Nevertheless, StCOL1 was degraded during the far-red application and *StSP5G* expression was reduced (Appendix, Fig. S6.4 and S6.5). Although *StSP5G* expression was a lot lower than in the long-day control treatment, *StSP6A* was only slightly upregulated. This experiment reinforces my earlier findings that coincidence of peak *StCOL1* expression and light is not the only inhibition on tuberization. Although far red can technically also be considered as light, most StCOL1 is degraded under far-red light (Abelenda *et al.*, 2016). Perhaps *StSP6A* expression was still low because the proposed unknown factor was still able to repress expression. A long day would lead to coincidence of light in the morning and the expression of this tuber repressing factor. Because far red is applied during this time, but repression on tuberization still exists, it may be the case that this additional factor can be stabilized by far-red light. As mentioned earlier, StCOL2 is stabilized by far-red light (Abelenda *et al.*, 2016), and may act as an additional factor controlling tuberization. Determining if silencing *StCOL2* induces or accelerates tuberization in long days and days with a night break, could include or rule out StCOL2 in this control. Interestingly Abelenda *et al.* (2016) did show that StCOL2 was able to induce *StSP5G*, indicating a possible additional control on tuberization through this second StCOL.

Day length extension with blue light
In Chapter 2, not only the effect of additional blue light throughout the day was tested, but also of the timing of blue light application. Extending short days with monochromatic blue light repressed tuberization, but blue-light extension was not as effective in this repression as white light (Chapter 2, Fig. 2.2A). Unsuccessful flower inhibition by blue light day-length extension, has been documented before in *Spinacea oleracea* and Chrysanthemum (Thomas and

Vince-Prue, 1997; Singh *et al.* 2013). The plants seem not to properly detect the blue light as a longer day. As mentioned earlier, white light has a stronger stabilizing function on StCOL1 than red or blue light alone (Abelenda *et al.*, 2016). Thus less stable StCOL1 in the blue-light extension may lead to a weaker inhibiting effect on tuberization than in a white-light extension. However, to stabilize StCOL1, the extended white or blue light has to coincide with *StCOL1* expression, but *StCOL1* is not expressed at the time of day-length extension in long or short days (ZT8-ZT16) (Chapter 3, Fig. 3.3A, M). Thus, ineffective tuber repression through blue-light day-extension is not likely to act through StCOL1.

Possibly, as I previously suggested for the EOD far-red treatment, PHYB may be involved in regulating *StCOL1* expression. When day length is extended with white light, PHYB remains active for the duration of a regular long day, leading to long-day *StCOL1* expression, which peaks in the morning, where light stabilizes StCOL1 and tuberization can be repressed. Blue light does not activate PHYB, and thus *StCOL1* expression may not be shifted so it still peaks at the end of the night, which does not repress tuberization. A simple way to determine if shifted *StCOL1* expression explains the ineffective repressing function of blue-light extension on tuberization, is by measuring *StCOL1* expression during day-length extensions with white or blue light.

Alternatively, the additional unknown tuber repressor mentioned in the previous section may explain the regulation of day-length extension. The expression of this unknown factor may depend on the dark period (Higuchi *et al.*, 2013), which may commence once PYHB is inactive. White light extension would activate PHYB and shift the expression of this factor to the morning, where coincidence with light could lead to an active/stable tuber repressor and repressed tuberization. Extension with blue light may not shift expression of the unknown factor, and allow the blue light of the extension to coincide with the expression of the unknown tuber repressing factor. Blue light may be less effective in stabilizing/activating the unknown tuber repressing factor than white light, as is the case for StCOL1 and StCOL2 both (Abelenda *et al.*, 2016). A hypothetical model explaining this control is illustrated in Fig. 6.7.

Although I suggest that PHYB is involved in measuring the dark period, which determines the start of expression of the unknown tuber repressor and perhaps also *StCOL1,* other photoreceptors may be crucial for this control. Yanovsky *et al.* (2000) showed that PHYA is involved in day-length perception and the entrainment of the circadian clock. However, an EOD far-red treatment

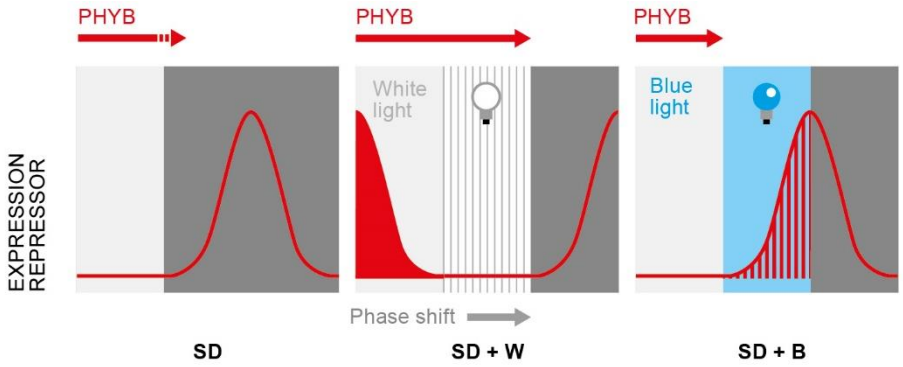


Figure 6.7. Hypothetical model showing day-length extension by white or blue light. This model shows how the expression of an unknown repressor of tuberization (red line) is delayed by day-length extension with white light (phytochrome B (PHYB) activated), but not by extension with blue light (PHYB deactivated). In a short day (SD), once PHYB is inactive through dark reversion, expression of the unknown tuber repressor can be induced and because of the long night the expression does not coincide with light (left pane). By extending the short day with white light (SD+W), PHYB activity is extended, causing the expression of the tuber repressing factor to start later (middle pane). Due to the shorter night, the expression coincides with light at beginning of the day, leading to activation/stabilization of tuber repressing factor, and repression of tuberization. Extending the day length with blue light (SD+B) (right pane), does not extend PHYB activity or affect the expression phase of the unknown repressor, allowing the blue light of the day-length extension to coincide with the expression of the tuber repressor. In this case the blue light may be less effective than white light in activating/stabilizing the tuberization repressor, leading to a weaker repression of tuberization.

would not function in deactivating PHYA, as it does PHYB (Casal, 2014). Thus, perhaps both PHYA and PHYB are involved in day-length perception and controlling the phase of *StCOL1*. Alternatively, the EOD far-red effect may function through another mechanism than day- or night-length perception.

Control through *StCOL*

This thesis demonstrates that the present photoperiodic and light spectral regulation on tuberization, through coincidence of *StCOL1* expression and light, is not complete. I suggest a working model including an additional factor regulated by the dark period, which may explain some of the unexpected results found in this thesis.

Regulation of potato reproduction through assimilates

Regulation through the daily light integral

High daily light integrals accelerate potato flowering and tuberization

As discussed in the previous section, photoperiod and light spectrum did not affect flower bud appearance time, only the number of leaves formed before the inflorescence (and only in *S. andigena*). Chapter 4 demonstrates that the DLI does have a strong effect on flowering time. Potato flower bud appearance was accelerated under high DLIs under short and long days. Furthermore, the number of leaves formed before the inflorescence was reduced under high DLIs, indicating a faster flower initiation (Almekinders and Struik, 1996). Although a change in the DLI in combination with a change in other climatic factors has been shown to affect flower initiation (Firman *et al.,* 1991), a direct positive effect of DLI on potato flower initiation has not been demonstrated until now.

While flower bud appearance time is an indication for flower initiation time, the possibility remains that flower bud development is also taken into account. Once flower initiation occurs, the flower buds develop to some extent, before they become macroscopically visible. Therefore, the leaf number is a more reliable measure for flower initiation. Consequently, it cannot be said with certainty that the flower initiation in days, is accelerated by high DLI, even though the flower bud appearance is accelerated. Nevertheless, I expect accelerated flower initiation to take place under higher DLIs. Scanning electron microscope photos of the shoot apical meristem of *S. andigena* have been made (Bergonzi *et al.,* unpublished), which indicate flower initiation occurs after four weeks (28 days) in plants grown in 8-hour day lengths with 300 $\mu mol \cdot m^{-2} \cdot s^{-2}$ light (DLI 8.6 $mol \cdot m^{-2}$). Based on the findings in this thesis, the flower bud appearance occurs at 27 days when the DLI is increased to 11.5 $mol \cdot m^{-2}$ under short days (Chapter 4, Fig. 4,1A). These findings imply that high DLI not only accelerates the flower bud appearance, but also the actual flower initiation. More scanning electron microscope photos of the shoot apical meristem under different DLIs could confirm this acceleration.

Although it was not presented in the research chapters of this thesis, tuberization was also accelerated by high DLI (Appendix, Exp. S1). Accelerated

tuberization under higher light intensities had been demonstrated before (Demagante and van der Zaag, 1988; Turner and Ewing, 1988). However, it had not yet been shown that a high DLI could induce tuberization in long days in the obligate short-day *S. andigena,* until now. These findings will be discussed in more detail below.

Though light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$) and DLI ($mol \cdot m^{-2} \cdot d^{-1}$) are difficult to separate, I suggest that the effect of high light (high intensity and high DLI) on flowering and tuberization time acts through the DLI and not the light intensity. When combining several day lengths, light intensities and DLIs, the effect of the DLI was most evident (Chapter 4, Discussion). The DLI has been proposed to regulate flowering in plants through assimilate formation (Thomas, 2006). Assimilates are known to affect potato reproduction (Ewing and Struik, 1992; Chincinska *et al.*, 2008), making DLI-mediated regulation of potato reproduction through assimilates plausible. Light intensity, on the other hand, does not have a clear mechanism how it could affect potato reproduction. Although photoreceptors have been found to sense differences in light intensities (Casal, 2000; Trupkin *et al.*, 2014; Ballaré and Pierik, 2017), it has not been demonstrated that differences among higher light intensities are distinguished (for instance between 200 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as tested in Chapter 4). Furthermore, it does not make sense in an evolutionary perspective that plant reproduction would respond to changes in light intensity (Bernier and Périlleux, 2005). Natural light intensity fluctuates continuously throughout the day. If plants would respond to a doubling of light intensity as a cue to start flowering, it would not help a plant to time its reproduction adequately. An environmental cue like photoperiod or DLI is more reliable, as it can tell the plant something about the environment in which it will start reproduction. This reasoning, in combination with the results found in Chapter 4, makes the DLI and thus assimilates, the best candidate through which high light can control potato flowering and tuberization.

High daily light integral does not regulate flowering and tuberization time through the photoperiodic controlled StSP3D and StSP6A

Assimilates can regulate flowering time in plants by inducing *FT* (Bouché *et al.*, 2016). However, Chapter 4 demonstrates that although *StSP3D* was upregulated by high DLIs, the accelerated flowering time was not caused by this upregulation. Flower initiation in the transgenic lines silenced in *StSP3D* was still accelerated

under high DLIs (faster flower bud appearance and less leaves formed before the inflorescence) (Chapter 4, Fig. 4.5). One exception was the *StSP3D RNAi#5* line, which had the same amount of leaves in the low and high light treatment in short days (SD200-SD400). However, considering flowering in SD200 was accelerated (less leaves), compared to the wild type, the leaf number in this transgenic line at SD200 may not be representative.

The accelerated flowering under high DLI in the *StSP3D RNAi* plants, suggests that the DLI accelerates flowering through a pathway that does not involve *StSP3D*. Interestingly, the high DLI may also accelerate tuberization independently of the photoperiodically regulated *StSP6A*. Although tuberization was accelerated in short days, and the high DLI led to upregulated *StSP6A* expression (Chapter 5, Appendix, Fig. S5.2), the high DLI treatment in long days also induced tuberization in *S. andigena* (Appendix, Fig. S6.1). This is surprising, as long-day conditions normally repress *StSP6A* expression and tuberization (Chapter 3, Fig. 3.2, Fig. 3.3O). Perhaps the high DLI in long days led to upregulated *StSP6A*, which induced tuberization. However, the treatment with high DLI did not induce *StSP6A* in long days, as can be seen in Appendix, Fig. S5.2, Chapter 5. Moreover, plants silenced in *StSP6A* could still to tuberize in the highest DLI treatment, in long days (Appendix, Fig. S6.2). Thus, not only can high DLI accelerate tuberization, but it may allow tuber induction, independently of *StSP6A*, circumventing the strong photoperiodic control on tuberization.

Considering assimilates are increased in the high DLI treatments (Chapter 4), assimilates may be able to control flowering and tuberization in potato, independently of *StSP3D* and *StSP6A.* Assimilates are known to regulate tuberization (Rodríguez-Falcón *et al.*, 2006) and it was even suggested that high sucrose concentrations may mask the effect of other control on tuberization (Prat, 2010). Chincinska *et al.* (2008) suggested sucrose may control tuberization through *FT* (*StSP6A*) regulation. Furthermore, it was suggested that a sucrose transporter StSUT4, could act on tuberization by affecting the photoperiodic pathway and *StSP6A* (Chincinska *et al.*, 2013). Nevertheless, the findings in this thesis give a first indication that high DLI (perhaps mediated through sucrose) may induce tuberization and flowering independently of *StSP6A* and *StSP3D*.

How can assimilates control potato reproduction independently of StSP3D and StSP6A?

Although a strong correlation between flowering time and sucrose concentration in the leaves was not found in Chapter 4, high DLIs did increase the sucrose

concentration in the plant. However, if sucrose does not act through *StSP3D* and *StSP6A,* how then? In Arabidopsis, sucrose affects flowering, in part through the activation of a flower inducing transcription factor *LEAFY* (*LFY*), which acts downstream of *FT* in the shoot apical meristem (Eriksson *et al.,* 2006). Also, sugars may induce flowering through the regulation of microRNAs miR156 and miR172, which are a class of non-coding, single stranded RNAs able to regulate gene expression (Matsoukas *et al.*, 2012; Yu *et al.,* 2015). *MiR172* overexpression accelerates flowering in several species (Albani and Coupland, 2010). MiR172 increases in the absence of miR156, and sugars are thought to repress miR156 (Yu *et al.*, 2015). Although the miRNA pathway has been shown to act on *FT* (Zhou and Wang, 2013), several examples show miRNAs are able to induce flowering independently of *FT*. MiR156 inhibits *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, which promote flowering by activating three flower controlling transcription factors *LFY*, *FRUITFULL* (*FUL*), and *APETALA1* (*AP1*) that all act downstream of *FT* (Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). Furthermore, Wahl *et al.* (2013) show that T6P, a proxy for sucrose concentration in the plant (Lunn *et al.*, 2006), is capable of directly controlling expression of *SPLs* in the shoot apical meristem. In potato a similar control may allow for flower induction independently of *StSP3D*. Plants overexpressing *miR156* were not able to flower (Bhogale *et al.*, 2014). Additionally, overexpression of *miR172* accelerated flower initiation (Martin *et al.*, 2009), even in long days when *StSP3D* expression is known to be low. Although it is not yet known if the control by miRNAs acts through *StSP3D*, measuring *StSP3D* expression in *miRNA* transgenic lines in short and long days may confirm or rule out this possibility. MiRNAs are also known to affect tuberization. Plants overexpressing *miR172* were able to tuberize in long days (Martin *et al.,* 2009). Overexpression of *miR156* reduced *miR172* expression (Bhogale *et al.*, 2014). If sugars can repress *miR156* in potato, this could be a way for the DLI to accelerate tuberization. However, as is the case for *StSP3D,* it is still unknown if miR172 acts through *StSP6A* or via an alternative route (Natarajan *et al.,* 2017).

MiRNAs are known to be involved in the age pathway in plants (Yu *et al.*, 2015). Although not studied in more detail in this thesis, miRNAs have an important role in the environmental regulation of potato reproduction. The importance of miRNAs in the control of tuberization has been recently reviewed (Natarajan *et al.* 2017) and discovering how miRNAs are involved in potato

flowering under different light treatments may advance the current knowledge on the regulation behind this process.

Competition between two modes of reproduction

The tuberization signal StSP6A represses flower bud development

Tuberization and flowering in potato are suggested to be competing processes, and competition is thought to act through the mutual need for assimilates to sustain tuber growth or flower bud development and berry set (Almekinders and Struik, 1996). The strong tuber sink may "drain" assimilates from the shoot to sustain tuber development, impairing flowering (Turner and Ewing, 1988). However, it has also been suggested that the pull from a strong sink is not the reason of impaired flowering, but that physiological changes in tuber-inducing conditions impair flowering (Turner and Ewing, 1988). Chapter 5 shows that competition between flowering and tuberization in the tested genotypes is regulated by the tuberization signal StSP6A. Removing the tuber sink through stolon pruning or grafting onto tuber-less rootstocks was not able to improve flower bud development. In the absence of these tuber sinks, assimilates still may have been directed towards alternative non-floral sinks in the form of aerial stolons and tubers formed directly on the stem (Chapter 5, Fig. 5.8). Therefore, removal of the tuber sink did not automatically lead to more assimilates for flower bud development. In contrary, by repressing the tuberization signal *StSP6A* in short days, flower bud development in *S. andigena* was improved (Chapter 5, Fig. 5.6). Furthermore, high *StSP6A* expression in CE3027 transgenic plants inhibited flower bud development in long days (Chapter 5, Fig. 5.7). As flower initiation occurred before tuberization, the inhibiting effect of the tuberization signal was expected to act only on flower bud development (Chapter 5), which took place after flower initiation. This was confirmed by the fact that flower bud appearance in *StSP6A* silenced lines was observed at the same time in short and long days (Chapter 5).

A surprising finding in Chapter 5 was that the non-tuberizing *S. etuberosum* rootstocks actually impaired flower bud development, opposed to not affecting flower bud development at all. Furthermore, a tuberizing rootstock (CE3027) improved *S. etuberosum* flowering. An explanation was discussed in Chapter 5, involving an increase in FTs which would improve flowering. However, a more practical explanation not discussed in Chapter 5 could be that

the *S. etuberosum* was not a good rootstock. Due to its zig-zagged stem with short internodes, the graft-union may have set incompletely, impeding proper assimilate flow through the stem in grafts with *S. etuberosum* rootstocks. Although the quality of the rootstock may have affected flower bud development, the experiments where stolons were removed also showed that removing the tuber sink did not improve flower bud development. These combined results strengthen our conclusions that not the tuber sink, but the tuberization signal impairs flower bud development.

The findings in Chapter 5 do not dismiss the possibility that removing the tuber sink can improve flower bud development in some genotypes, which has been documented in the past (Thijn, 1954; Jessup, 1958). It could be the case that a direct repressing effect of StSP6A is genotype specific, which will be further discussed below. Furthermore, successful flowering and tuberization do take place simultaneously in some genotypes. In Chapter 5 it was mentioned that there is a huge variation in flowering success between genotypes (European Cultivated Potato Database, https://www.europotato.org). However, successful flowering in these genotypes does not rule out an impairing role of StSP6A. First, good flowering does not mean that even better flowering cannot be obtained if *StSP6A* is repressed. It would be interesting to find out if repression of *StSP6A* in genotypes with successful flowering improves flower development even further. Second, as suggested by Pallais *et al.* (1987), the earliness of tuberization may play a role in the flowering success, thus the impairing role of StSP6A may not have an effect if flower development is already at an advanced stage before *StSP6A* is expressed.

In the absence of the tuberization signal, flower bud development may be a short-day process

As previously mentioned, *StSP3D* is expressed at high levels in short days, but is lowly expressed in long days. Flower initiation, measured in the number of leaves, was accelerated in short days in *S. andigena*, indicating flowering may be under weak photoperiodic control, even though the flower bud appearance time was not affected. Conversely, flower bud development, often measured through anthesis (open flowering stage), is considered to be a long-day process (Driver and Hawkes, 1943; Zafar, 1955; Pallais, 1987; Almekinders, 1992; Ewing and Struik, 1992; Almekinders and Struik, 1994; Macháčková *et al.*, 1998; Markarov, 2002; Schittenhelm *et al.*, 2004). The results in this thesis confirm these findings

(Chapter 5). However, it is also demonstrated that by repressing *StSP6A* expression, short-day flower bud development can match and even surpass long-day flowering (Chapter 5, Fig. 5.5). Thus, short days may actually be inductive for flower bud development, but competition with the tuberization signal StSP6A masks this photoperiodic effect. Perhaps photoperiodic control of *StSP3D* is not crucial for flower initiation, but for flower bud development.

FTs StSP3D and StSP6A may control flower and tuber development by redirecting assimilates

From the literature and my own results, it is likely that both StSP6A and StSP3D play key roles in controlling flower bud development. Reducing StSP6A also strongly reduced the formation of aerial stolons and tubers growing directly from the stem (Chapter 5). Thus, StSP6A may be responsible for directing assimilates towards the formation of tuberization structures. This hypothesis is reinforced by the finding that StSP6A can interact with the sucrose efflux transporter StSWEET11, which together may have a role in redirecting the flow of assimilates towards tuberization (Abelenda *et al.,* unpublished). Interestingly, StSP3D has also been found to interact with StSWEET11 (Bergonzi *et al.* unpublished), which together may have a role in redirecting assimilates towards flowering. However, there is no experimental evidence for this redirection of assimilates and a mechanism for how this directionality would take place has yet to be proposed. Nevertheless, StSP6A may repress flower bud development by obstructing the assimilate flow towards the shoot apical meristem. There is experimental evidence that StSP6A is transported to the stolons (Navarro *et al.*, 2011), indicating directionality connected with StSP6A transportation. It would thus be important to investigate whether StSP3D is directed towards the shoot apex in potato. If this is indeed the case, directionality of these FTs may explain assimilate flow direction. While little is known about the mechanism behind the transport of FTs in plants, there is good evidence of long distance movement of these proteins through the vasculature, towards the plant apical meristems (Corbesier *et al.*, 2007). A flaw in this theory in potato, however, is that StSP3D may not be a mobile signal, as was discussed earlier. Discovering if StSP3D is a mobile signal, would be a crucial step in understanding both flower initiation and flower bud development.

With the current knowledge on interactions between a sugar transporter and StSP6A or StSP3D, combined with findings in Chapter 5, a hypothetical

working model explaining competition between reproduction modes in potato is proposed (Fig. 6.8). In short-day conditions, *StSP6A* and *StSP3D* are both expressed in the leaves, but transcript levels of *StSP3D* are lower than those of *StSP6A* (Navarro *et al.*, 2011). StSP6A and StSP3D may compete with each other for the binding of StSWEET11. However, because StSP6A is more abundant than StSP3D, the chance that StSP6A will interact with StSWEET11 is greater thus assimilates would preferentially be directed towards the tuber sink (Fig. 6.8A). In long days, *StSP6A* is not expressed, while low levels of *StSP3D* expression may still take place (Abelenda *et al.*, 2014) (Chapter 4, Fig. 4.5). In the absence of StSP6A, even at low levels, StSP3D can interact with StSWEET11 and assimilates may then be directed towards the shoot apex, thereby maintaining flower bud development (Fig. 6.8B). However, when *StSP6A* is repressed in short days, the

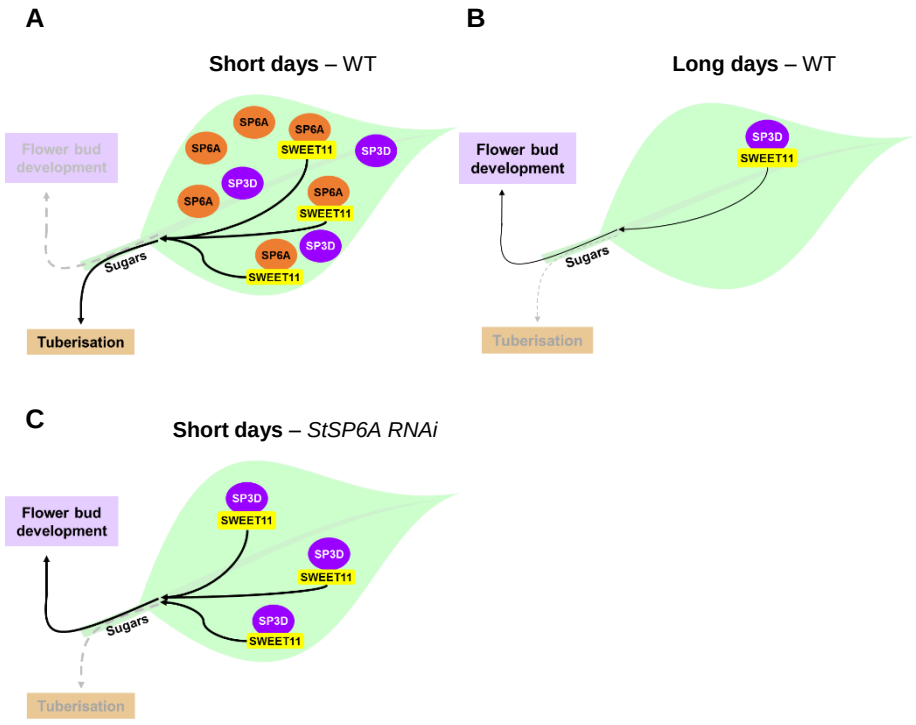


Figure 6.8. Hypothetical model on assimilate redirection through interaction between StSP3D, StSP6A and sugar transporter StSWEET11. (A) In short days StSP6A is more abundant than StSP3D, allowing StSP6A to interact with StSWEET11 and direct assimilates toward tuberization. (B) In long days, StSP6A is not present and only a little StSP3D is available to direct assimilates toward flower bud development. (C) In short days in plants silenced in *StSP6A,* StSP6A is absent and StSP3D is more abundant than in long days. Therefore, more StSP3D can interact with StSWEET11 and direct assimilates toward flower bud development, leading to improved flower bud development.

relatively highly expressed *StSP3D* is likely to facilitate more interaction between StSP3D and StSWEET1 and potentially direct more assimilates to the shoot apex, allowing even better flower bud development (Fig. 6.8C).

This model fits with the finding that high DLI in short days (*StSP6A* expressed), and the associated increase in assimilates, improves flower bud development (Chapter 5). Even though the fraction of assimilates directed to the shoot apex remains the same, increasing the total amount of acquired assimilates may allow more assimilates to reach the shoot apex. The flower bud development under higher DLIs is further improved by repressing *StSP6A* (Chapter 5), because the fraction of assimilates directed to the shoot apex may be increased. In contrast, removing the tuber sink in induced plants (*StSP6A* expressed) will not change the total amount of the assimilates in the plant, nor the fraction of assimilates directed towards the shoot apex. Thus, flower bud development is not improved. However, when *StSP6A* is repressed, the total amount of assimilates is not increased, but the fraction of assimilates directed towards the shoot apex may increase, improving flower bud development. Further research will be necessary to confirm this model and clarify if and how interaction between StSWEET11 and StSP6A/StSP3D can actually direct the assimilate flow.

As mentioned previously, some studies demonstrate a positive effect on flower bud development when removing the tuber sink. Potato breeders have used several methods to improve potato flowering, for instance by planting potatoes on stones, which allows them to easily remove the tubers (Thijn, 1954). How can a model involving assimilate redirection by StSP6A and StSP3D explain the positive effects of tuber-sink removal found in the past? Perhaps the level of interaction between StSWEET11 and both StSP6A and StSP3D is genotype specific. In some genotypes the assimilate distribution may depend less on StSWEET11-FT interactions and more on sink strength. In this case, removing the tuber sink may allow for redistribution of assimilates throughout the whole plant, including the shoot apex, instead of directional assimilate flow only towards tuber structures. To test this hypothesis, the tuberization and flowering signal interaction with StSWEET11 could be measured in genotypes where tuber-sink removal improves flowering.

In summary, findings in this thesis, combined with the knowledge that StSP6A and StSP3D can interact with a sugar transporter, make it possible that competition in potato occurs through the active direction of assimilate flows to

either tuberization or flowering. Although competition between flowering and tuberization was expected to act through assimilates, by means of the sink strength of the tubers (Almekinders and Struik, 1996), the findings in this thesis suggest a more active control of assimilates takes place, which is regulated by the tuberization and flowering signal. In this case, assimilates would not only play a role in inducing tuberization and flowering in potato, but also in sustaining these processes.

Using light to control reproduction in potato

Light as an environmental switch

The results in this thesis indicate that light can be used to control potato reproduction. The (molecular) mechanisms behind this control are slowly being elucidated. This increased knowledge can be used to implement light as a regulator of potato reproduction in practice.

Light spectrum in the form of far-red light can be applied at the end of a long day to accelerate tuberization (in modern varieties that tuberize in long-day conditions). When tuberization is not yet required and it is desired that the potato plant grows larger, the day length can be increased as longer days favor shoot growth. A high DLI (by increasing light intensity) can be applied to accelerate both flowering and tuberization.

For favorable flowering conditions, a combination of a high DLI to accelerate flower initiation, long days to improve flower bud development, and blue light to delay tuberization, may be ideal. For improved tuberization, high DLI and end-of-day far-red light in long days may allow for high yield and accelerated tuberization. A short day would also accelerate tuberization, but the light intensity would have to be considerably increased to acquire the same amount of assimilates as in a long day, to promote high tuber yields. It must be noted that although some light conditions may accelerate tuberization, very early tuberization is not desired as it may impair shoot growth, which eventually leads to less assimilate formation and thus less assimilates to ensure good tuber yields (Werner, 1935).

An optimal environment for either tuberization or flowering?

Although the right combination of light spectrum, photoperiod and DLI may improve flowering or tuberization, a perfect environment for either tuberization or flowering does not exist, because the two processes are intertwined. Light factors accelerating flower initiation, like high DLI, also accelerate tuberization, which in turn inhibits flower bud development. Nevertheless, extra emphasis can be put on tuberization or flowering when either is required, and light creates an ideal environmental control switch for this. In the case of plant breeding or potato propagation, performed in greenhouses or climate chambers, the controlled environments offer the opportunity to adjust light settings. However, for potato production, which is in the open field, light may be trickier to regulate. Applying additional light next to the already available sunlight, would be a technical and financial challenge. A night break could be applied with additional lights if days are short but plants are required to continue shoot growth for an extended period prior to initiating tuberization. In the case of an end-of-day far-red treatment, to some extent this already takes place in the field, as afternoon sun contains a lower red to far-red ratio than midday sun, due to the angle of the sun compared to the surface of the earth (Thomas and Vince-Prue, 1997; Franklin and Whitelam, 2007, Hogewoning *et al.* 2010). However, reducing the ratio even further with far-red LEDs spread across the field may still have an additional effect. The plant density could also be increased, which lowers the red to far-red ratio as well (reviewed in: Ballaré and Pierik, 2017). However, this would be paired with a lower light intensity and DLI per plant, which delays both flowering and tuberization. Nevertheless, light treatments can be implemented to control potato reproduction to favor flowering or tuberization. Finally, it is important to realize that genotype specificity is common, as has been shown in various experiments in this thesis (Chapter 2, 4 and 5) and thus it must be tested whether the light effects found in this thesis also occur in the varieties that are used in practice.

In summary, this thesis shows how light can be used to control tuberization and flowering and describes the processes that underlie this control. With this information, light will be able to be implemented as a regulator of potato reproduction in the near future.

Appendix

Supplementary experiment 1. High-light accelerated tuberization.

Not only was the effect of high DLI on flower initiation studied, but also the effect on tuberization time was measured. We determined if higher DLIs accelerated tuberization and if this acceleration was controlled through upregulated *StSP6A* expression

Materials and methods
S. andigena and CE3027 wild-type plants and the *StSP6A* RNAi plants (*StSP6A RNAi* #1 and *StSP6A RNAi* #13) from the experiments described in Chapter 4 (Exp. 1-2) and Chapter 5 (Exp. 3) were not only scored for flowering time, but were also used to determine tuberization time. Tuberization time was scored by carefully removing the soil around the stolons and checking the stolon tips for swelling, three times a week. As opposed to *StSP3D* in Chapter 4, *StSP6A* expression was determined, to find out if tuberization under high light was controlled through the photoperiod pathway and *StSP6A*. Expression of *StSP6A* under low and high light in *S. andigena* wild type and *StSP6A RNAi* lines in an *S. andigena* background, was determined and described in the Appendix of Chapter 5, Fig. S5.2.

Results
Increasing the DLI accelerated tuberization in both CE3027 and *S. andigena.* In short days this acceleration was only significant in CE3027, although *S. andigena* did show a trend towards faster tuberization under high light (Fig. S6.1). *S. andigena*, was able to tuberize in long days, but only when the DLI was high. This was not a strong tuberization response, as only 31% (not shown) of the plants grown under the highest DLI tuberized. In the *S. andigena* transgenic lines silenced in *StSP6A,* tuberization was repressed in most treatments (Fig. S6.2). However, the highest DLI treatment in long days resulted in tuberization. Though, just like in the wild type, this tuberization only took place in some plants (<40%).

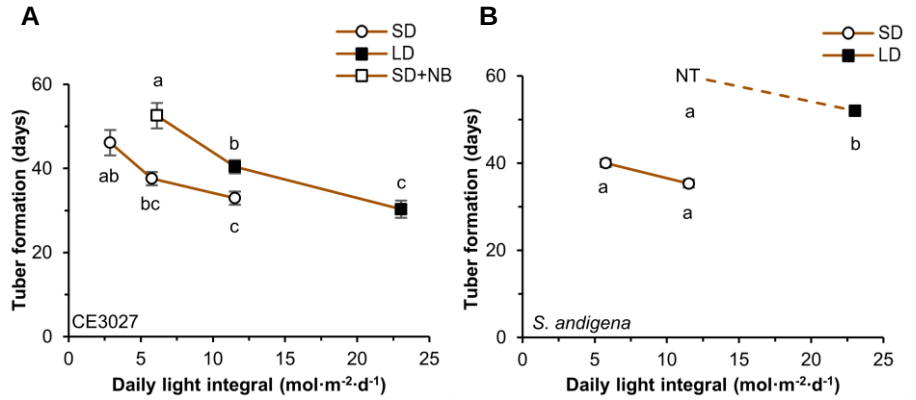


Figure S6.1. Tuberization time in light treatments with different daily light integrals (DLI). Time from transplanting until the start of tuberization in CE3027 (A) and *S. andigena* (B) is given. Plants were grown in different DLIs in short days (SD, 8/16 hours light/dark, open circles), in long days (LD, 16/8 hours light/dark, closed squares) and in a short day with a night break of 15 minutes in the middle of the night (SD+NB, 8/16 hours light/dark, open squares). Error bars are the standard error of difference (ANOVA). Different letters indicate significant differences between light treatments (Fisher's protected LSD test, α = 0.05). NT = no tuberization, the dashed line in (B) indicates that tuberization was accelerated under high DLI, however, as the treatment with a low DLI in long days did not tuberize at the time of harvest, it is unknown how this line would run.

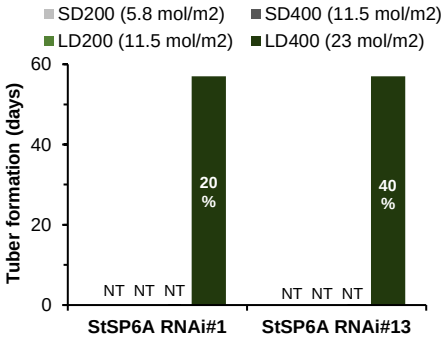


Figure S6.2. Tuberization time in transgenic lines. The effect of the daily light integral (DLI) is given on tuberization time in *StSP6A RNAi#1* and *StSP6A RNAi#13,* both in an *S. andigena* background. Plants were grown in short days (SD, 8/16 hours light/dark) under 200 µmol·m^{-2}·s^{-1} and 400 µmol·m^{-2}·s^{-1} light, and in long days (LD, 16/8 hours light/dark) under 200 µmol·m^{-2}·s^{-1} and 400 µmol·m^{-2}·s^{-1} light. The DLI is indicated after the light treatment. NT = no tuberization at time of harvest. The percentages indicate how many plants tuberized.

Supplementary experiment 2. Timing far-red light to induce tuberization.

To determine if far-red light applied during the peak expression of *StCOL1* could induce tuberization in a long day in *S. andigena*, an additional experiment was performed.

Materials and methods

Three treatments were applied to *S. andigena* plants: (1) a short day (8/16 h day/night), (2) a long day (16/8 h day/night) (both as described in Chapter 3), and (3) a long day where during the first three hours of the light period only far red was supplied (Fig. S6.3). Far red was provided by Philips GreenPower LED (production module 120cm far red) with an intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a PSS (phytochrome stationary state) value of 0.11 (where 1 is fully active PHYB and 0 is fully inactive PHYB). The light spectrum given throughout the day was identical to light in the long- and short-day treatments (Philips GreenPower LED production module 120cm DeepRedWhite-2012). However, because far-red light is not photosynthetically active radiation, a higher intensity of red/white light was applied to make the DLI (photoperiod x light intensity) identical in all treatments (light intensities of 185 $\mu mol \cdot m^{-2} \cdot s^{-1}$ compared to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the long day and 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the short day). This far-red treatment was performed in the same climate chamber as the long-day control treatment and the growing protocol is described in detail in Chapter 3.

Genotypes used were a wild-type *S. andigena* and a transgenic line in the *S. andigena* background, overexpressing *StCOL1* with an HA-tag (35S::StCOL1-HA). These genotypes were also used and described in Chapter 3. The wild type was sampled for gene expression analysis and the overexpressing line was sampled for protein detection. The sampling protocol, qPCR analysis, western blotting and data analysis were performed as described in Chapter 3.

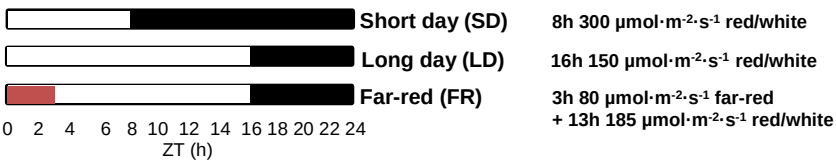


Figure S6.3. Light treatments. Plants were grown in three LED-light treatments. A short day, a long day and a long day with pure far-red light applied for the first three hours of the day. The white bar indicates red/white control light and the red bar indicates pure far-red light. Light intensities are indicated and all treatments received the same daily light integral (8.6 $mol \cdot m^{-2}$). ZT = Zeitgeber time (hours after light stimulation).

Results

The far-red treatment in long days did not induce tuberization, as was the case for the long-day control (Chapter 3). Far-red light applied in the beginning of the day, during peak *StCOL1* expression degraded StCOL1 protein during this time (Fig. S6.4). In the long day this StCOL1 was clearly present in the beginning of the light period. The far-red treatment not only led to StCOL1 degradation from ZT0-3, but also to a repression of *StSP5G* compared to long days (Fig. S6.5H). Although *StSP5G* was partially repressed in the far-red treatment, *StSP6A* expression was still very low compared to the short-day treatment (Fig. S6.5I, C), which did lead to tuberization (Chapter 3). *StSP6A* expression was upregulated compared to the long-day treatment. The far-red treatment did not affect *StCOL1* expression, only StCOL1 stability.

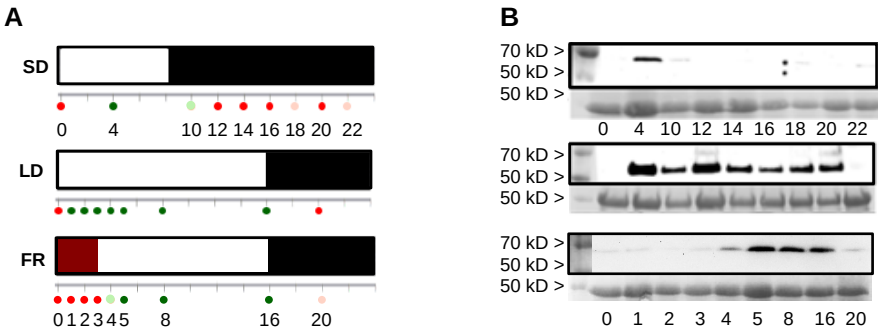


Figure S6.4. **Western blot analysis of StCOL1 protein.** Protein detection was done in 35S:StCOL1L1-HA plants after four weeks of growing on soil. The far-red treatment is compared to a short and long-day treatment (treatment details in Fig. S6.3). (A) A schematic representation of StCOL1 protein levels is given (red: no StCOL1, pink: little StCOL1, light green: some StCOL1, green: clear StCOL1 presence), the numbers indicate the hours after the light treatment started. (B) A Western blot. A ponceau stain is shown as a loading control. Absence of a band shows that StCOL1 protein is degraded at the given time. Protein presence of the short- and long-day treatment has already been shown in Chapter 3 (Fig. 3.4), and is only depicted here as a comparison.

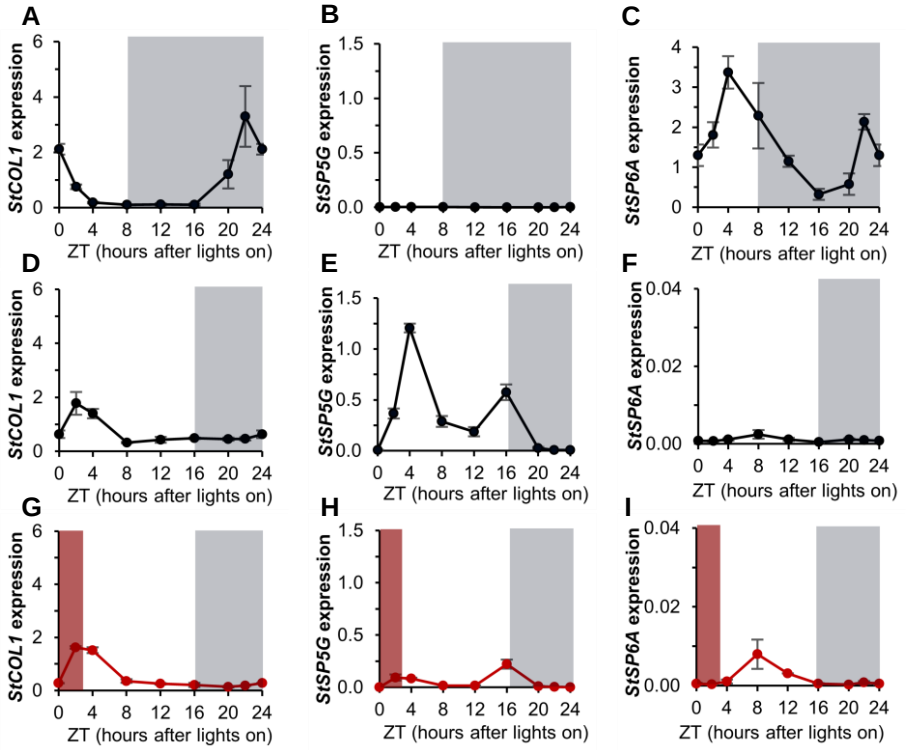


Figure S6.5. Gene expression in light treatments with or without added far-red light. *StCOL1*, *StSP5G* and *StSP6A* expression after four weeks of growing in (A-C) short days (8/16 hours light/dark), (D-F) in long days (16/8 hours light/dark) and (G-I) in a treatment with far-red light in the beginning of the light period (3/13/8 hours far-red/light/dark). ZT= Zeitgeber time, hours after lights on. Error bars indicate standard errors of the mean. The long and short day expression patterns have already been shown in Chapter 3 and are only used here as a comparison.

References

Abdel-Wahab, A., & Miller, J. (1963). Re-evaluation of some techniques and their effect on stimulating flowering in four Irish potato varieties in Louisiana. *American Potato Journal*, **40,** 53–57.

Abelenda, J.A., Navarro, C., & Prat, S. (2011). From the model to the crop: genes controlling tuber formation in potato. *Current Opinion in Biotechnology*, **22**, 287–292.

Abelenda, J.A., Navarro, C., & Prat, S. (2014). Flowering and tuberization: a tale of two nightshades. *Trends in Plant Science*, **19**, 115–122.

Abelenda, J.A., Cruz-oro, E., Franco-zorrilla, J.M., & Prat, S. (2016). Potato StCONSTANS-like1 suppresses storage organ formation by directly activating the *FT-like StSP5G* Repressor. *Current Biology*, **26**, 872–881.

Adams, S.R., Pearson, S., Hadley, P., & Patefield, W. (1999). The effects of temperature and light integral on the phases of photoperiod sensitivity in *Petunia x hybrida*. *Annals of Botany*, **83**, 263–269.

Albani, M.C., & Coupland, G. (2010). Comparative analysis of flowering in annual and perennial plants. *Current Topics in Developmental Biology*, **91**, 323–348.

Almekinders, C.J.M. (1992). The effect of photoperiod on flowering and TPS production in the warm tropics. *Potato Research*, **35**, 433–442.

Almekinders, C.J.M., & Struik, P.C. (1994). Photothermal response of sympodium development and flowering in potato (*Solanum tuberosum* L.) under controlled conditions. *Netherlands Journal of Agricultural Science*, **42**, 311–329.

Almekinders, C.J.M., & Struik, P.C. (1996). Shoot development and flowering in potato *(Solanum tuberosum* L.). *Potato Research*, **39**, 581–607.

Andrés, F., & Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics*, **13**, 627–639.

Atwell, B.J., Kriedemann, P.E., & Turnbull, C.G.N. (1999). Book: *Plants in action: adaptation in nature, performance in cultivation,* MacMillan Education AU.

Ballaré, C.L., & Pierik, R. (2017). The shade-avoidance syndrome: multiple signals and ecological consequences. *Plant, Cell and Environment*, **40**, 2530-2543

Banerjee, A.K., Chatterjee, M., Yu, Y., Suh, S., Miller, W.A., & Hannapel, D.J. (2006). Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. *The Plant Cell*, **18**, 3443–3457.

Batutis, E.J., & Ewing, E.E. (1982). Far-red reversal of red light effect during long-night induction of potato (*Solanum tuberosum* L.) tuberization. *Plant Physiology*, **69**, 672–674.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., & Lejeune, P. (1993). Physiological signals that lnduce flowering. *The Plant Cell*, **5**, 1147–1155.

Bernier, G., & Périlleux, C. (2005). A physiological overview of the genetics of flowering time control. *Plant Biotechnology Journal*, **3**, 3–16.

Bhogale, S., Mahajan, A.S., Natarajan, B., Rajabhoj, M., Thulasiram, H.V., & Banerjee, A.K. (2014). MicroRNA156: A potential graft-transmissible microRNA That modulates plant architecture and tuberization in *Solanum tuberosum* ssp. andigena. *Plant Physiology*, **164**, 1011–1027.

Bolouri Moghaddam, M.R., & van den Ende, W. (2013). Sugars, the clock and transition to flowering. *Frontiers in Plant Science*, **4**, 1–6.

Borthwick, H.A., & Cathey, H.M. (1962). Role of phytochrome in control of flowering of Chrysanthemum. *The University of Chicago Press*, **123**, 155–162.

Bouché, F., Lobet, G., Tocquin, P., & Périlleux, C. (2016). FLOR-ID: an interactive database of flowering-time gene networks in Arabidopsis thaliana. *Nucleic Acids Research*, **44**, 1167–1171.

Cao, K., Yan, F., Xu, D., Ai, K., Yu, J., Bao, E., & Zou, Z. (2018). Phytochrome B1-dependent control of *SP5G* transcription is the basis of the night break and red to far-red light ratio effects in tomato flowering. *BMC Plant Biology*, **18**, 1–12.

Casal, J. J. (2000). Phytochromes, cryptochromes, phototropin: photoreceptor interactions in plants. *Photochemistry and Photobiology*, **71**, 1–11.

Casal, J.J. (2013). Photoreceptor signaling networks in plant responses to shade. *Annual Review of Plant Biology*, **64**, 403–427.

Casal, J.J., Candia, A.N., & Sellaro, R. (2014). Light perception and signalling by phytochrome A. *Journal of Experimental Botany*, **65**, 2835–2845.

Cashmore, A.R., Jarillo, J.A., Wu, Y., & Liu, D. (1999). Cryptochromes : blue light receptors for plants and animals. *Science*, **284**, 760–766.

Celis-Gamboa, C. (2002). PhD thesis: *The life cycle of the potato (Solanum tuberosum L.): from crop physiology to genetics*. Plant Breeding.

Chapman, H.W. (1958). Tuberization in the potato plant. *Physiologia Plantarum*, **11**, 215–224.

Chen, H., Rosin, F., Prat, S., & Hannapel, D. (2003). Interacting transcription factors from the three-amino acid loop extension superclass regulate tuber formation. *Plant Physiology*, **132**, 1391–1404.

Chincinska, I.A, Liesche, J., Krügel, U., Michalska, J., Geigenberger, P., Grimm, B., & Kühn, C. (2008). Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response. *Plant Physiology*, **146**, 515–528.

Chincinska, I., Gier, K., Krügel, U., Liesche, J., He, H., Grimm, B., Harren, F.J.M., Cristescu, S.M., Kühn, C. (2013). Photoperiodic regulation of the sucrose transporter StSUT4 affects the expression of circadian-regulated genes and ethylene production. *Frontiers in Plant Science*, **4**, 1–12.

Cho, S. K., Sharma, P., Butler, N.M., Kang, I., Shah, S., Rao, A.G., & Hannapel, D.J. (2015). Polypyrimidine tract-binding proteins of potato mediate tuberization through an interaction with StBEL5 RNA. *Journal of Experimental Botany*, **66**, 6835–6847.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science*, **316**, 1030–1033.

Craver, J.K., Miller, C.T., Williams, K.A., & Bello, N.M. (2014). Ultraviolet radiation affects intumescence development in ornamental sweetpotato (I*pomoea batatas*). *HortScience*, **49**, 1277–1283.

Demagante, A.L., & Van der Zaag, P. (1988). The response of potato (Solanum spp.) to photoperiod and light intensity under high temperatures. *Potato Research*, **31**, 73–83.

Dijken, A J.H. Van, Schluepmann, H., & Smeekens, S.C.M. (2004). Arabidopsis Trehalose-6-Phosphate Synthase 1 is essential for normal vegetative growth and transition to flowering. *Plant Physiology*, **135**, 969–977.

Driver, C.M., & Hawkes, J.G. (1943). Book: *Photoperiodism in potato*. Imperial Bureau of Plant Breeding and Genetics, Cambridge.

Eriksson, S., Böhlenius, H., Moritz, T., & Nilsson, O. (2006). GA 4 is the active gibberellin in the regulation of *LEAFY* transcription and Arabidopsis floral initiation. *The Plant Cell*, **18**, 2172–2181.

Ewing, E.E., & Struik, P.C. (1992). Tuber formation in potato: induction, initiation, and growth. *Horticultural Reviews*, **14**, 89–198.

Fankhauser, C. (2001). The phytochromes, a family of red/far-red absorbing photoreceptors. *The Journal of Biological Chemistry*, **276**, 11453–11456.

Firman, D.M., Brien, P.J.O., & Allen, E.J. (1991). Leaf and flower initiation in potato (*Solanum tuberosum*) sprouts and stems in relation to number of nodes and tuber initiation. *Journal of Agricultural Science*, **117**, 61–74.

Fixen, K.R., Thomas, S.C., & Tong, C.B.S. (2012). Blue light inhibition of tuberization in a day-neutral potato. *Journal of Plant Growth Regulation*, **31**, 342–350.

Friedman, M., McDonald, G.M., & Filadelfi-Keszi, M. (1997). Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. *Critical Reviews in Plant Sciences*, **16**, 55–132.

Fu, J., Yang, L., & Dai, S. (2014). Identification and characterization of the CONSTANS-like gene family in the short-day plant *Chrysanthemum lavandulifolium*. *Molecular Genetics and Genomics*, **290**, 1039–1054.

Garner, W.W., & Allard, H.A. (1923). Further studies in photoperiodism, the response of the plant to relative length of day and night. *Journal of Agricultural Research*, **23**, 871–959.

González-Schain, N.D., & Suárez-López, P. (2008). CONSTANS delays flowering and affects tuber yield in potato. *Biologia Plantarum*, **52**, 251–258.

González-Schain, N.D., Díaz-Mendoza, M., Żurczak, M., & Suárez-López, P. (2012). Potato CONSTANS is involved in photoperiodic tuberization in a graft-transmissible manner. *The Plant Journal*, **70**, 678–690.

Gregory, L.E. (1956). Some factors for tuberization in the potato plant. *Amererican Journal of Botany*, **43**, 281–288.

Hannapel, D. (2007). Signalling the induction of tuber formation. In: Vreugdenhil D, Bradshaw J, Gebhardt, C., Govers, F., Taylor, M., MacKerron, D., Ross, H. (Eds), *Potato Biology and Biotechnology: Advances and Perspectives*, Elsevier Science, pp. 237–256

Hannapel, D.J., Sharma, P., Lin, T., & Banerjee, A.K. (2017). The multiple signals that control tuber formation. *Plant Physiology*, **174**, 845–856.

Havelange, A., & Bernier, G. (1983). Partial floral evocation by high irradiance in the long-day plant *Sinapis alba*. *Physiologia Plantarum*, **59**, 545–550.

Hermsen, J.G.T., Taylor, L.M., van Breukelen, E.W.M., & Lipski, A. (1978). Inheritance of genetic markers from two potato dihaploids and their respective parent cultivars. *Euphytica*, **27**, 681–688.

Higuchi, Y., Sumitomo, K., Oda, A., Shimizu, H., & Hisamatsu, T. (2012). Day light quality affects the night-break response in the short-day plant chrysanthemum, suggesting differential phytochrome-mediated regulation of flowering. *Journal of Plant Physiology*, **169**, 1789–1796.

Higuchi, Y., Narumi, T., Oda, A., Nakano, Y., Sumitomo, K., Fukai, S., & Hisamatsu, T. (2013). The gated induction system of a systemic floral inhibitor, antiflorigen, determines obligate short-day flowering in chrysanthemums. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 17137–17142.

Hirsch, C.N., Hirsch, C.D., Felcher, K., Coombs, J., Zarka, D., Van Deynze, A., de Jong, W., Veilleux, R.E., Jansky, S., Bethke, P., Douches, D.S., Buell, C.R. (2013). Retrospective view of North American potato (*Solanum tuberosum* L.) breeding in the 20th and 21st centuries. *Genes Genomes Genetics*, **3**, 1003–1013.

Hogewoning, S.W., Douwstra, P., Trouwborst, G., van Ieperen, W., & Harbinson, J. (2010). An artificial solar spectrum substantially alters plant development compared with usual climate room irradiance spectra. *Journal of Experimental Botany*, **61**, 1267–1276.

Horridge, J.S., & Cockshull, K.E. (1989). The effect of the timing of a night-break on flower initiation in C*hrysanthemum morifolium* Ramat. *Journal of Horticultural Science*, **64**, 183–188.

Horton, D.E., & Anderson, J.L. (1992). Potato production in the context of the world and farm economy. In: Harris, P.M. (Ed), *The Potato Crop,* Springer, Dordrecht, pp. 794–815.

Hosaka, K., & Hanneman, Jr., R.E. (1998). Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense.* 1. Detection of an S locus inhibitor (Sli) gene. *Euphytica*, **99**, 191–197.

Imaizumi, T., & Kay, S.A. (2006). Photoperiodic control of flowering : not only by coincidence. *Trends in Plant Science*, **11**, 550–558.

Ishikawa, R., Tamaki, S., Yokoi, S., Inagaki, N., Shinomura, T., Takano, M., & Shimamoto, K. (2005). Suppression of the floral activator Hd3a is the principal cause of the night break effect in rice. *The Plant Cell*, **17**, 3326–3336.

Izawa, T., Takahashi, Y., & Yano, M. (2003). Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and Arabidopsis. *Current Opinion in Plant Biology*, **6**, 113–120.

Jackson, S.D., & Prat, S. (1996). Control of tuberization in potato by gibberellins and phytochrome B. *Physiologia Plantarum*, **98**, 407–412.

Jackson, S.D., Heyer, A., Dietze, J., & Prat, S. (1996). Phytochrome B mediates the photoperiodic control of tuber formation in potato. *The Plant Journal*, **9**, 159–166.

Jackson, S., & Thomas, B. (1997). Photoreceptors and signals in the photoperiodic control of development. *Plant, Cell & Environment*, **20**, 790–795.

Jackson, S.D. (1999). Multiple signaling pathways control tuber induction in potato. *Plant Physiology*, **119**, 1–8.

Jansky, S.H., Charkowski, A.O., Douches, D.S., Gusmini, G., Richael, C., Paul, C., Spooner, D.M., Novy, R.G., de Jong, H., de Jong, W.S., Bamberg, J.B., Thompson, L., Bizimungu, B., Holm, D.G., Brown, C.R., Haynes, K.G., Vidyasagar, R.,Veilleux, R.E., Miller, J.C., Bradeen, J.M., Jiang, J.M. (2016). Reinventing potato as a diploid inbred line – Based Crop. *Crop Science*, **11**, 1–11.

Jessup, R.J. (1958). Potato breeding in New South Wales. *The Journal of the Australian Institute of Agricultural Science*, **6**, 21–26.

Jones, H.A., & Borthwick, H.A. (1938). Influence of photoperiod and other factors on the formation of flower primordia in the potato. *The American Potato Journal*, **15**, 331–336.

Karimi, M., Inze, D., & Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science*, **7**, 193–195.

Kinet, J. M. (1977). Effect of light conditions on the development of the inflorescence in tomato. *Scientia Horticulturae*, **6**, 15–26.

King, R., & Bagnall, D. (1996). Photoreceptors and the photoperiodic response controlling flowering of Arabidopsis. *Cell and Developmental Biology*, **7**, 449–454.

Kloosterman, B., Abelenda, J.A., Gomez, M. del M.C., Oortwijn, M., de Boer, J.M., Kowitwanich, K., Horvath B.M., van Eck H.J., Smaczniak C., Prat S., Visser R.G.F., Bachem C.W.B. (2013). Naturally occurring allele diversity allows potato cultivation in northern latitudes. *Nature*, **495**, 246–250.

Krantz, F.A. (1939). Incidence and inheritance of pollen sterility in the potato. *Journal of Agricultural Research*, **58**, 593–602.

Krauss, A., & Marschner, H. (1984). Growth rate and carbohydrate metabolism of potato tubers exposed to high temperatures. *Potato Research*, **27**, 297–303.

Lee, R., Baldwin, S., Kenel, F., McCallum, J., & Macknight, R. (2013). *FLOWERING LOCUS T* genes control onion bulb formation and flowering. *Nature Communications*, **4**, 2884.

Lejeune, P., Bernier, G., Requier, M., & Kinet, J. (1993). Sucrose increase during floral induction in the phloem sap collected at the apical part of the shoot of the long-day plant *Sinapis alba* L. *Planta*, **32**, 71–74.

Levy, Y.Y., & Dean, C. (1998). The transition to flowering. *The Plant Cell*, **10**, 1973–1989.

Li, J., Li, G., Wang, H., & Deng, X.W. (2011). Phytochrome signaling mechanisms. In: *The Arabidopsis Book,* American Society of Plant Biologists, pp. 1–26.

Lifschitz, E., & Eshed, Y. (2006). Universal florigenic signals triggered by FT homologues regulate growth and flowering cycles in perennial day-neutral tomato. *Journal of Experimental Botany*, **57**, 3405–3414.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P., Eshed, Y. (2006). The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6398–6403.

Lindhout, P., Meijer, D., Schotte, T., Hutten, R.C.B., Visser, R.G.F., & van Eck, H.J. (2011). Towards F1 hybrid seed potato breeding. *Potato Research*, **54**, 301–312.

Liu, H., Yu, X., Li, K., Klejnot, J., Yang, H., Lisiero, D., & Lin, C. (2008). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. *Science*, **322**, 1535–1540.

Lunn, J.E., Feil, R., Hendriks, J.H.M., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M. R., Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana. Biochemical Journal*, **148**, 139–148.

Macháčková, I., Konstantinova, T.N., Sergeeva, L.I., Lozhnikova, V.N., Golyanovskaya, S.A., Dudko, N. D., Eder, J., Aksenova, N.P. (1998). Photoperiodic control of growth, development and phytohormone balance in *Solanum tuberosum*. *Physiologia Plantarum*, **102**, 272–278.

Markarov, A.M. (2002). Causes of flowering of long-day potato species under short-day and cold-night conditions. *Russian Journal of Plant Physiology*, **49**, 465–469.

Martin, A., Adam, H., Díaz-Mendoza, M., Zurczak, M., González-Schain, N.D., & Suárez-López, P. (2009). Graft-transmissible induction of potato tuberization by the microRNA miR172. *Development*, **136**, 2873–2881.

Martínez-García, J.F., Virgós-Soler, A., & Prat, S. (2002). Control of photoperiod-regulated tuberization in potato by the Arabidopsis flowering-time gene *CONSTANS*. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 15211–15216.

Massa, G.D., Kim, H., Wheeler, R.M., & Mitchell, C.A. (2008). Plant productivity in response to LED lighting. *HortScience*, **43**, 1951–1956.

Matsoukas, I.G., Massiah, A.J., & Thomas, B. (2012). Florigenic and antflorigenic signaling in plants. *Plant & Cell Physiology*, **53**, 1827–1842.

Medzihradszky, M., Bindics, J., Ádám, É., Viczián, A., Klement, É., Lorrain, S., Gyula, P., Mérai, Z., Fankhauser, C., Medzihradszky, K.F., Kunkel, T., Schäfer, E., Nagy, F. (2013). Phosphorylation of phytochrome B inhibits light-induced

signaling via accelerated dark reversion in Arabidopsis. *The Plant Cell*, **25**, 535–544.

de Montaigu, A., Giakountis, A., Rubin, M., Tóth, R., Cremer, F., Sokolova, V., Porri, A., Reymond, M., Weinig, C., Coupland, G. (2015). Natural diversity in daily rhythms of gene expression contributes to phenotypic variation. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 905–910.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobaoco tissue cultures. *Physiologia Plantarum*, **15**, 473–497.

Natarajan, B., Bhogale, S., Banerjee, A.K., (2017). The essential role of microRNAs in potato tuber development : a mini review. *Indian Journal of Plant Physiology*, **22**, 401–410.

Navarro, C., Abelenda, J.A, Cruz-Oró, E., Cuéllar, C.A, Tamaki, S., Silva, J., Shimamoto, K., Prat, S. (2011). Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature*, **478**, 119–123.

Navarro, C., Cruz-Oró, E., & Prat, S. (2015). Conserved function of FLOWERING LOCUS T (FT) homologues as signals for storage organ differentiation. *Current Opinion in Plant Biology*, **23**, 45–53.

Odell, J., Nagy, F., & Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature*, **313**, 810–812.

Ouzounis, T., Rosenqvist, E., & Ottosen, C.O. (2015). Spectral effects of artificial light on plant physiology and secondary metabolism: A review. *HortScience*, **50**, 1128–1135.

Pallais, N. (1987). True potato seed quality. *Theoretical and Applied Genetics*, **73**, 784–792.

Pearce, S., Shaw, L.M., Lin, H., Cotter, J.D., Li, C., & Dubcovsky, J. (2017). Night-break experiments shed light on the Photoperiod1-mediated flowering. *Plant Physiology*, **174**, 1139–1150.

Pin, P.A., & Nilsson, O. (2012). The multifaceted roles of FLOWERING LOCUS T in plant development. *Plant, Cell & Environment*, **35**, 1742–1755.

Plantenga, F.D.M., Siakou, M., Bergonzi, S., Heuvelink, E., Bachem, C.W.B., Visser, R.G.F., & Marcelis, L.F.M. (2016). Regulating flower and tuber formation in potato with light spectrum and day length. *Acta Horticulturae*, **1134**, 267–276.

Potato Genome Sequencing Consortium. (2011). Genome sequence and analysis of the tuber crop potato. *Nature*, **475**, 189–195.

Prat, S. (2010). Hormonal and daylength control of potato tuberization. In: Davies, P.J. (Ed), *Plant Hormones,* Springer, Dordrecht, pp. 574–596.

Rodríguez-Falcón, M., Bou, J., & Prat, S. (2006). Seasonal control of tuberization in potato: conserved elements with the flowering response. *Annual Review of Plant Biology*, **57**, 151–180.

Ruddat, A., Schmidt, P., Gatz, C., Braslavsky, S.E., Gärtner, W., & Schaffner, K. (1997). Recombinant type A and B phytochromes from potato. Transient absorption spectroscopy. *Biochemistry*, **36**, 103–111.

Sager, J., Smith, W., Edwards, J., & Cyr, K. (1988). Photosynthetic efficiency and phytochrome photoequilibria determination using spectral data. *Transactions of the Asae*, **31**, 1882-1889.

Sarlikioti, V., De Visser, P.H.B., Buck-Sorlin, G.H., & Marcelis, L.F.M. (2011). How plant architecture affects light absorption and photosynthesis in tomato: Towards an ideotype for plant architecture using a functionalstructural plant model. *Annals of Botany*, **108**, 1065–1073.

Sawa, M., Nusinow, D.A., Kay, S.A., & Imaizumi, T. (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science*, **318**, 261–266.

Schafleitner, R., Ramirez, J., Jarvis, A., Evers, D., Gutierrez, R., & Schurrah, M. (2011). Adaptation of the potato crop to changing climates. In: Yadav, S.S., Redden, R.J., Hatfield, J.L., Lotze-Campen, H., Hall, A.E. (Eds), *Crop adaptation to climate change,* John Wiley & Sons, pp. 287–297**.**

Schittenhelm, S., Menge-Hartmann, U., & Oldenburg, E. (2004). Photosynthesis, carbohydrate metabolism, and yield of phytochrome-B-overexpressing potatoes under different light regimes. *Crop Science*, **44**, 131–143.

Schmittgen, T.D., & Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, **3**, 1101–1108.

Seternes, T., Tonheim, T.C., Myhr, A.I., & Dalmo, R.A. (2016). A plant 35S CaMV promoter induces long-term expression of luciferase in Atlantic salmon. *Scientific Reports*, **6**, 1–6.

Singh, M., van Ieperen, W., & Heuvelink, E. (2013). Effect of LEDs on flower bud induction in Chrysanthemum morifolium cv. Zembla. *HortFlora Research Spectrum*, **2**, 185–188.

Slater, J.W. (1964). Mechanisms of tuber initiation. In: Ivins, J.D., Milthope, F.L. (Eds), *The growth of potato*, Butterwoyhs, London, pp. 114–120.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **51,** 49–81.

Smith, H., & Whitelam, G.C. (1997). The shade avoidance syndrome: multiple responses mediated by multiple phytochromes. *Plant, Cell and Environment*, **20,** 840–844.

Spreitzer, R.J. (1993). Genetic dissection of Rubisco structure and function. *Annual Review of Plant Physiology and Plant Molecular Biology*, **44,** 411–434.

Song, Y.H., Ito, S., & Imaizumi, T. (2010). Similarities in the circadian clock and photoperiodism in plants. *Current Opinion in Plant Biology*, **13**, 594–603.

Song, Y.H., Smith, R.W., To, B.J., Millar, A.J., & Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilisation in photoperiodic flowering. *Science*, **336**, 1045–1050.

Song, Y.H., Shim, J.S., Kinmonth-Schultz, H.A, & Imaizumi, T. (2014). Photoperiodic flowering: time measurement mechanisms in leaves. *Annual Review of Plant Biology*, **66**, 441-464

Soyk, S., Müller, N.A., Park, S.J., Schmalenbach, I., Jiang, K., Hayama, R., Zhang, L., Van Eck, J., Jiménez-Gómez, J.M., Lippman, Z.B. (2017). Variation in the

flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. *Nature Genetics*, **49**, 162–168.

Srikanth, A., & Schmid, M. (2011). Regulation of flowering time: all roads lead to Rome. *Cellular and Molecular Life Sciences*, **68**, 2013–2037.

Sukhotu, T., Kamijima, O., & Hosaka, K. (2005). Genetic diversity of the Andean tetraploid cultivated potato (*Solanum tuberosum* L. subsp. andigena Hawkes) evaluated by chloroplast and nuclear DNA markers. *Genome*, **64**, 55–64.

Sun, J., Wang, H., Ren, L., Chen, S., Chen, F., & Jiang, J. (2017). CmFTL2 is involved in the photoperiod- and sucrose-mediated control of flowering time in chrysanthemum. *Horticulture Research*, **4**, 1–6.

Sweetlove, L.J., Kossmann, J., Riesmeier, J.W., Trethewey, R.N., & Hill, S.A. (1998). The control of source to sink carbon flux during tuber development in potato. *The Plant Journal*, **15**, 697–706.

Taylor, S. (2010). A practical guide to publishing RT-qPCR Data that conform to the MIQE Guidelines. *American Biotechnology Laboratory*, **28**, 14–19.

de Temmerman, L. (2007). Response to the environment: carbon dioxide. In: Vreugdenhil D, Bradshaw J, Gebhardt, C., Govers, F., Taylor, M., MacKerron, D., Ross, H. (Eds), *Potato Biology and Biotechnology: Advances and Perspectives*, Elsevier Science, pp. 395–413.

Thijn, G.A. (1954). Observations on flower induction with potatoes. *Euphytica*, **3,** 28–34.

Thomas, B., & Vince-Prue, D. (1997). *Photoperiodism in plants*. Academic Press.

Thomas, B. (2006). Light signals and flowering. *Journal of Experimental Botany*, **57**, 3387–3393.

Trupkin, S.A., Legris, M., Buchovsky, A.S., Belén, M., Rivero, T., & Casal, J.J. (2014). Phytochrome B nuclear bodies respond to the low red to far-red ratio and to the reduced irradiance of canopy shade in Arabidopsis. *Plant Physiology*, **165**, 1698–1708.

Tsuji, H., Taoka, K.I., & Shimamoto, K. (2011). Regulation of flowering in rice: Two florigen genes, a complex gene network, and natural variation. *Current Opinion in Plant Biology*, **14**, 45–52.

Turner, A.D., & Ewing, E.E. (1988). Effects of photoperiod, night temperature, and irradiance on flower production in the potato. *Potato Research*, **31**, 257–268.

Visser, R. G. F. (1991). Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In: Lindsey, K. (Ed.), *Plant Tissue Culture Manual*. Springer, Netherlands, pp. 301–309.

Wahl, V., Ponnu, J., Schlereth, A., Arrivault, S., Langenecker, T., Franke, A., Feil, R., Lunn, J. E., Stitt, M., Schmid, M. (2013). Regulation of flowering by Trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science*, **339**, 704–707.

Wang, J., Czech, B., & Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell*, **138**, 738–749.

Wang, S., Zhang, S., Wang, W., Xiong, X., Meng, F., & Cui, X. (2015). Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. *Plant Cell Reports*, **34**, 1473–1476.

Weinheimer, W.H., & Woodbury, G.W. (1966). Effects of grafting and solanum understocks on flower formation and abscission of flowers and fruits in the Russet Burbank potato. *American Potato Journal*, **43**, 453–457.

Werner, H. (1935). The effect of temperature, photoperiod and nitrogen level upon tuberization in the potato. *The American Potato Journal*, **12**, 274–280.

Xu, X., van Lammeren A.A., Vermeer, E., & Vreugdenhil, D. (1998). The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation in vitro. *Plant Physiology*, **117**, 575–584.

Yamaguchi, A., Wu, M., Yang, L., Wu, G., Poethig, R.S., & Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of *LEAFY*, *FRUITFUL*, and *APETALA1*. *Developmental Cell*, **17**, 268–278.

Yanovsky, M.J., Izaguirre, M., Wagmaister, J.A,, Gatz, C., Jackson, S.D., Thomas, B., & Casal, J.J. (2000). Phytochrome A resets the circadian clock and delays tuber formation under long days in potato. *The Plant Journal: For Cell and Molecular Biology*, **23**, 223–232.

Yu, S., Lian, H., & Wang, J. (2015). Plant developmental transitions: the role of microRNAs and sugars. *Current Opinion in Plant Biology*, ***27,*** 1–7.

Zafar, M.A. (1955). Application of certain hormones to prevent flower abscission in two potato (*Solanum tuberosum*) varieties. *American Potato Journal*, **32**, 283–292.

Zaheer, K., & Akhtar, M.H. (2016). Potato production , usage , and nutrition — a review. *Critical Reviews in Food Science and Nutrition*, **56**, 711–721.

de Zeeuw, D. (1954). The influence of the leaf on flowering. *Mededelingen van de Landbouwhogeschool Te Wageningen*, **54**, 1–44.

Zhou, C., & Wang, J. (2013). Regulation of flowering time by microRNAs. *Journal of Genetics and Genomics*, **40**, 211–215.

Zuo, Z., Liu, H., Liu, B., Liu, X., & Lin, C. (2011). Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis. *Current Biology*, **21**, 841–847.

Summary

Potato plants can reproduce sexually through the formation of flowers, berries and seeds, and asexually through the formation of tubers, commonly called the potatoes. Potato tuberization is a well-studied process, but flowering in potato is seldom the focus of scientific research. However, due to the recent possibility of using hybrid breeding techniques in potato, true potato seeds can be used as starting material as well as the tubers. These developments have caused some of the attention to shift from tuberization to flowering. In this thesis tuberization and flowering were studied simultaneously.

The environment can be used as a flexible switch to control reproduction in potato, steering it towards flowering when seeds are required for breeding or propagation, or towards tuberization when tubers are required for propagation or potato production. Light is a convenient environmental switch, as we are able to manipulate the spectrum (color), photoperiod (day length), light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$) and the daily light integral (DLI, $mol \cdot m^{-2} \cdot d^{-1}$). The objectives of this study were to 1) quantify the effects of photoperiod, light spectrum, light intensity and the DLI on tuberization and flowering time, 2) determine if tuberization and flowering compete and if so, how this is regulated, and to 3) discover which molecular mechanisms underlie regulation of tuberization and flowering by light.

Chapter 1 describes what is known about regulation of potato reproduction by light. Reports on the effect of light on potato tuberization and especially on potato flowering are often contradictory or information is lacking. In some cases several light factors are altered simultaneously, making it impossible to draw strong conclusions about a specific light factor. In this chapter, information from various researches was compared. This way, the effect of light on potato reproduction could be separated into an effect by light spectrum, photoperiod, intensity or DLI. Additionally, mechanisms underlying these effects were summarized and missing links were determined.

Chapter 2 determines how light spectrum (far-red and blue light) and photoperiod (long day vs. short day) can be used to regulate potato reproduction. In this chapter it is demonstrated that by applying far-red light, which deactivates PHYB, tuberization time was accelerated in long days. This

acceleration only took place in genotypes that were able to tuberize in long days (not obligate short-day genotypes). It was also shown that the addition of blue light to a short day, delayed tuberization. However, this effect was genotype specific. Finally, results in this chapter demonstrated that extending day length with low-intensity white light, delayed or even inhibited tuberization, while day-length extension with blue light was less effective in this inhibition. Neither far-red light, blue light nor day-length extension was found to affect the flower bud appearance time.

Chapter 3 determines whether an external coincidence model can explain how a night break (30 minutes of light in the dark period) inhibits tuberization. In the model plant Arabidopsis, photoperiodic flowering is controlled by coincidence of light and *CONSTANS (CO)* expression. In potato, a similar model has been hypothesized to explain photoperiodic tuberization. In this chapter it was investigated whether coincidence of light and potato *CO* (*StCOL1*) expression, could explain how a night break is able to inhibit short-day tuberization. By using night breaks that coincided with *StCOL1* expression in the night or not, it was demonstrated that coincidence between light and peak *StCOL1* expression does not always lead to repressed tuberization. This chapter suggests that there are additional factors involved in photoperiodic control of potato tuberization. In addition, it was shown that although flower bud appearance time is not affected by photoperiod, the number of leaves formed before the inflorescence in the genotype *S. andigena* decreases under shorter photoperiods.

Chapter 4 demonstrates that high DLI accelerates flowering time in potato. This acceleration was found in both short and long days, and was measured in the days until flower bud appearance, and in the number of leaves formed before the inflorescence (less leaves formed). A correlation between high DLI-accelerated flowering and plant carbohydrate concentration was tested, but not found. In this chapter plants silenced in the flowering time gene *StSP3D* were used to demonstrate that flowering is accelerated under high DLIs, independently of the flowering time gene *StSP3D*.

Chapter 5 focusses on competition between tuberization and flowering. In this chapter, the effect of tuberization on the flower bud development was determined. It was demonstrated that removing the tuber sink (by grafting or removing stolons) did not improve the flower development in tuber inducing conditions (short days), while silencing the tuberization signal *StSP6A* did. It was

also demonstrated that increasing *StSP6A* in long days impaired flower bud development.

Chapter 6 discusses how the above results fit into a general model. Light spectrum and photoperiod are part of the same control, as photoreceptor PHYB acts on StCOL1, which is a crucial part of the photoperiod pathway. Furthermore, It is proposed that PHYB may also repress tuberization by acting on a yet unknown factor, which acts downstream of *StCOL1*. It is discussed why the effect of DLI on flowering could be mediated through assimilates, even though a direct correlation between the increase of assimilates and the flower bud appearance time was not found. Furthermore, additional results were presented, demonstrating that high DLI-accelerated tuberization may be regulated independently of the photoperiodically controlled *StSP6A.* This chapter gives examples how assimilates could induce flowering and tuberization independently of *StSP3D* and *StSP6A.* Once tuberization and flowering are initiated, competition between the processes may take place. In this chapter a model is presented where StSP3D competes with StSP6A to direct assimilates towards either tuber or flower bud development.

This final chapter is concluded with practical applications for the results obtained in this PhD project. It is suggested how light could be used as a flexible regulator to manipulate potato reproduction.

Samenvatting

De aardappelplant kan zich zowel seksueel voortplanten door bloemen, bessen en zaden te vormen, of het kan zich aseksueel voortplanten, door knollen te vormen. Knolvorming in de aardappel is een goed bestudeerd proces, maar er is zelden aandacht voor de aardappelbloei in wetenschappelijke studies. Echter, dankzij nieuwe mogelijkheden om hybrideveredeling toe te passen in de aardappel, kunnen naast de knollen ook de zaden gebruikt worden voor vermeerdering. Door deze ontwikkelingen schuift wat van de aandacht van de knollen naar de aardappelbloemen. In dit proefschrift zullen beide reproductieprocessen tegelijk bestudeerd worden.

Omgevingsfactoren kunnen gebruikt worden om aardappelreproductie op een flexibele manier te reguleren. Zo kan de aardappelplant gestuurd worden om te bloeien, wanneer dit nodig is voor veredeling of zaadproductie, of om knollen te vormen, wanneer dit nodig is voor aardappelproductie of vermeerdering. Licht kan gebruikt worden om aardappelreproductie aan te sturen, omdat we het spectrum, de daglengte, de lichtintensiteit en de dagelijkse lichtsom kunnen variëren. De doelstellingen van deze studie waren om 1) de effecten van daglengte, lichtspectrum, lichtintensiteit en de dagelijkse lichtsom op knolvormingstijd en bloeitijd te kwantificeren, 2) te bepalen of knolvorming en bloei concurrerende processen zijn en zo ja, hoe deze concurrentie wordt gereguleerd, en tenslotte om 3) te ontdekken welke moleculaire mechanismen ten grondslag liggen aan de regulatie van de knolvorming en bloei door licht.

Hoofdstuk 1 beschrijft hoe aardappelreproductie wordt beïnvloed door licht. Informatie over de invloed van licht op knolvorming en vooral op aardappelbloei is vaak tegenstrijdig of incompleet. In sommige gevallen worden meerdere lichtfactoren tegelijk gevarieerd, waardoor het onmogelijk wordt om sterke conclusies te trekken over het effect van een specifieke lichtfactor. In dit hoofdstuk werden resultaten uit verschillende onderzoeken vergeleken. Hierdoor kon het effect van licht op aardappelreproductie opgesplitst worden in een effect door lichtspectrum, daglengte, lichtintensiteit of dagelijkse lichtsom. Daarnaast werden de belangrijkste achterliggende mechanismen samengevat, en werd er bepaald welke informatie nog mistte.

Hoofdstuk 2 toont aan hoe lichtspectrum (verrood en blauw licht) en de daglengte (lange dag versus korte dag) gebruikt kunnen worden om aardappelreproductie aan te sturen. In dit hoofdstuk wordt aangetoond dat het toedienen van verrood licht (dat PHYB deactiveert), de knolvorming in lange dagen versnelt. Deze versnelling werd alleen waargenomen in genotypen die sowieso knollen konden vormen in lange dagen. Daarnaast werd in dit hoofdstuk vastgesteld dat het toedienen van blauw licht in korte dagen de knolvorming kon remmen, maar dat dit effect specifiek voor het genotype was. Tot slot, werd aangetoond dat daglengteverlenging met laag intensiteit wit licht de knolvorming remt, maar daglengteverlenging met blauw licht hier minder effectief in is. Verrood licht, blauw licht, noch daglengte hadden invloed op de bloeitijd in aardappel (de tijd die nodig was tot het verschijnen van de eerste bloemknoppen).

Hoofdstuk 3 onderzoekt hoe een nachtonderbreking (30 minuten licht toegediend in het midden van de nacht) de knolvorming in korte dagen kan remmen. Bloei in het modelorganisme Arabidopsis wordt bepaald door de samenloop van *CONSTANS (CO)* expressie en licht. In het licht wordt CO gestabiliseerd en kan het, het bloeisignaal induceren. Het is voorgesteld dat een vergelijkbaar mechanisme in aardappel de knolvorming bepaalt. In dit hoofdstuk werd vastgesteld of de samenloop van *StCOL1* expressie en licht de werking van een nachtonderbreking kon verklaren. Door gebruik te maken van verschillende nachtonderbrekingen, die wel of niet samenvielen met *StCOL1* expressie in de nacht, werd aangetoond dat samenval van licht en *StCO* expressie niet altijd leidt tot onderdrukking van de knolvorming. In dit hoofdstuk werd voorgesteld dat er nog onbekende bijkomende factoren betrokken zijn in daglengte gereguleerde knolvorming. Daarnaast werd aangetoond dat de bloeitijd niet beïnvloed wordt door de daglengte, maar dat de hoeveelheid bladeren die gevormd worden voor de bloeizetting wel afnemen in korte dagen.

Hoofdstuk 4 laat zien dat een hoge dagelijkse lichtsom de bloeitijd in aardappel versnelt. Deze versnelling werd gevonden in zowel korte als lange dagen en werd gemeten in dagen tot het verschijnen van de eerste bloemknop, en in de hoeveelheid bladeren die voor de bloeizetting gevormd werd (minder bladeren bij een hoge dagelijkse lichtsom). Een verband tussen de assimilaten-concentratie in de plant en de snellere bloeitijd onder hogere dagelijkse lichtsom kon niet bewezen worden. In dit hoofdstuk werden transgene planten gebruikt waarin het gen voor het bloeisignaal *StSP3D* onderdrukt was. Hiermee kon

worden aangetoond dat versnelde bloei onder een hoge dagelijkse lichtsom niet wordt gereguleerd via *StSP3D.*

Hoofdstuk 5 richt zich op de concurrentie tussen knolvorming en de bloei. In dit hoofdstuk werd het effect van de knolvorming op de ontwikkeling van de bloemknoppen bepaald. Er werd aangetoond dat de bloeiontwikkeling wordt belemmerd in omstandigheden waar knolvorming wordt geïnduceerd. De resultaten lieten zien dat het verwijderen van de knol zelf, door te enten of stolonen te verwijderen, niet leidde tot verbeterde bloeiontwikkeling, terwijl het onderdrukken van het knolvormingssignaal *StSP6A* de bloemontwikkeling wel bevorderde. Bovendien werd aangetoond dat het verhogen van de *StSP6A* expressie de ontwikkeling van bloemknoppen onderdrukte.

Hoofdstuk 6 bespreekt hoe de bovengenoemde resultaten passen in een algemeen model. Lichtspectrum en daglengte maken deel uit van dezelfde pathway. De fotoreceptor PHYB wordt beïnvloed door het lichtspectrum en is betrokken bij de stabilisatie van StCOL1, dat een cruciaal onderdeel is van de daglengte pathway. Daarnaast werd in dit hoofdstuk gesuggereerd dat PHYB de knolvorming ook zou kunnen onderdrukken via een nog te definiëren factor, die down-stream van *StCOL1* functioneert. In dit hoofdstuk werd behandeld waarom het effect van de dagelijkse lichtsom op bloei gereguleerd zou kunnen zijn door assimilaten, hoewel er geen sterk verband te vinden was tussen een toename in plantassimilaten en de bloeitijd. Ook werden nieuwe resultaten besproken, die aangaven dat een versnelde knolvorming bij een hogere dagelijkse lichtsom mogelijk onafhankelijk gebeurt van de expressie van het knolvormingssignaal *StSP6A*. Vervolgens werd besproken hoe assimilaten mogelijk betrokken kunnen zijn bij bloei- en knolinductie onder hoge dagelijkse lichtsom, onafhankelijk van *StSP3D* en *StSP6A*. Daarnaast zijn assimilaten ook betrokken bij de verdere ontwikkeling van de bloemen en de knollen. In dit hoofdstuk werd een model geïntroduceerd waarin StSP6A en StSP3D met elkaar concurreren om de assimilaten in de plant naar ofwel de knolvorming, ofwel de bloei te sturen.

Dit laatste hoofdstuk wordt afgesloten met praktische toepassingen voor de resultaten behaald in dit proefschrift. Er wordt voorgesteld hoe licht kan worden gebruikt als een flexibele regelaar om de aardappelreproductie te sturen.

Acknowledgements

First of all I would like to state the obvious. There is no way I could have finished this PhD project by myself. Many people contributed to this research, in the form of supervision, advice, practical help or just by keeping me sane. I would like to start with the last, and probably the most important person that kept me motivated to keep going. Stefan, sorry to have been a grumpy root these past four and a half years. Thank you for your patience and surprisingly good advices for someone not in "this field". You always helped me by listening, making me see the other side of problems and standing by me when times where rough (het komt wel goed!). Also thank you for helping me with night measurements and harvests when nobody else was available (and sorry for being so strict, it was all in the name of science!). I love how you now explain the workings of far-red light to your friends and sound like a real plant nerd :). Also a special professional thanks for designing the beautiful cover and chapter spreads of this thesis and for helping me with the text design and figures.

Of course this project was not only mine to start with. Thank you Leo Marcelis, Ep Heuvelink, Christian Bachem, Richard Visser, Solynta, Philips (Signify), Beekenkamp and Incotec for coming up with this great research project. Leo, you are a great supervisor, every time I thought I was stuck and was dreading our weekly talk, you had a solution and I could continue with renewed motivation. You always took the time to help me and took my ideas seriously. Ep, thank you for your practical advice and of course your knowledge on statistics. I don't know what I would have done without you. Christian, thank you for your unlimited patience and willingness to explain the molecular work to me. I know it must have been frustrating to explain molecular biology to an ecologist at the beginning, but this has been extremely valuable to me and I have learnt so much from you. Richard, always the first to send comments on my papers. Thank you, your comments were very valuable for the quality of my work.

A special thanks to Sara Bergonzi, the other half of the FlowerPot project! You were always willing to explain the complex molecular mechanisms you were working on, even if it took me a long time to fully understand. You showed me how to run a qPCR and guided me through the lab (in which I was very lost at the

time). Our discussions on our daily train ride from Amsterdam to Wageningen were very insightful. Although our often puzzling results were very frustrating to me at times, you always stayed positive and curious to find a solution behind the results. I really enjoyed our joint experiments in the tiny climate chambers and the 24-hour measurements spent camping in Klima. I know your help was indispensable to me and can only hope I was able to help you a bit too. Abe, although you weren't officially involved in the project you were a crucial part of the team. The "molecular mastermind" who could always answer all our questions and come up with explanations for anything. I couldn't follow you half the time, but I learnt so much from you. When Sara wasn't around, you were my go-to guy in the lab. Thank you for being so patient and a special thanks for explaining how to run a western blot. I still have the pictures I took paparazzi-style showing every step of the long process.

Furthermore I would like to thank the companies involved in the research. First Pim, Michiel, Julia, Sjaak and Marcela of Solynta. You and your company are an inspiration. Thanks for our monthly discussions and keep up the good work with Solynta! To all other research partners that were present at our half yearly meetings, Henry at Incotec, Angela, Frank and Jos at Beekenkamp and Celine at Philips (Signify), thank you so much for your interest in the project, your critical questions and the passion in your profession you brought to every meeting. I learnt a lot from you. Also a special thanks to Celine, Esther, Eugen and Sjoerd at Philips (Signify) for the LEDs we used in this project and for advice on the lighting plan.

A thank you to all the technicians that helped with this project: Marian and Isolde in the Plant Breeding lab. Thank you so much for helping out when I couldn't do it myself. And also for being so optimistic and a joy to work with. You made me feel welcome in the Plant Breeding molecular and tissue culture labs where I only occasionally made an appearance. Francisca, thank you so much for saving me at the end of my project. After Abe left, you helped me set up the western blot in the HPP lab and taught me the tricks of the trade. Not only did your excellent supervision help make my Western Blotting a success (finally), but you made me feel comfortable in the lab and thanks to you I now really enjoy doing molecular work. Juriaan, I don't know what I would have done without you. In the very end, when I did not have time left to finish western blotting and gene expression analyses, you did the qPCR for me. Thank you so much. Not only were we able to finish on time, but the quality of your work was so much better than I

could have done myself. Keep up the good work finishing your master's degree, I'm sure you are going to be an amazing PhD candidate. Arjen, thanks for your patience with me in the lab when explaining the HPLC (and when I thought I broke the vacuum pump). Joke and Menno, thanks for your help with my experiment when I was on holidays. Of course a special thanks to the Klima team, Gerrit, Taede, David, Rohan and Rinie. Thanks for being so flexible with my weird climate chamber set-ups and always taking the time to come up with creative solutions. Also for generally being "gezellig" and making working in Klima feel more like fun than work. Thanks to other Unifarm staff members for help in the greenhouse (special thanks to André and Erik for help with installations) and in the dry lab (Herman, Wim, Gerrit, thanks for being so flexible with my harvests which always took longer than expected). Also I would like to thank Priscila for spending a whole day helping me to make grafts, Elias for doing photosynthesis measurements on the potatoes and Yongran and Sharath for helping me with my harvest.

A huge thank you to the students I was allowed to supervise. Melpo, I supervised you when I just started my PhD and you probably taught me more than I taught you. Thank you for your hard work and dedication and your motivated attitude, and just being a joy to work with. I could really rely on you and am sure you will successfully finish your PhD in the near future. Joep, thanks for your tremendous help in the climate chamber. Your hard work led to a nice bachelor thesis and the results from this work were very insightful to us.

My colleagues at HPP, you made this PhD a success for me. The most valuable part of this project was the friendships I obtained while in this group. First of all my paranymphs Ana and Priscila, thank you for supporting me during the defense and for being good friends to me throughout and after the PhD. I'm sure you both will look gorgeous on that stage! To my small PhD office mates Evelien, Xixi and Haris. Travelling the 1.5 hours to Wageningen wasn't too bad if you guys were there. Lots of laughs, stupid jokes, and on lucky days nice snacks. My big PhD office mates Ningyi, Alejandro, Maarten, Arian, Rachel, Dorthe, Yongran, Fahrizal, Yutaka, Ying, Sharath, Kim, Hua. I will have such fond memories of our times together, thanks for making the HPP group a happy group. The post docs scattered around the other rooms: Aina, Dália, Habtamu and Sander, thanks for being there for good advice and for adding to the good atmosphere. My former PhD neighbor, turned staff member, turned post doc, Elias, thanks for being a mentor when I just arrived and thanks for your strict but

good scientific advise (500 words per day!). David, although you were not officially part of the HPP group it felt like you were. Thanks for hanging around, listening, and your friendship (and for the amba!). I would also like to thank the PhD's I used to sit with that graduated or started a career :) Nikos, Geert, Pavlos, Tao, Sasan, Graham, Craig, Jonathan, Daniel, René. Thanks for accepting me and letting me into your "man cave" which the PhD office was when I started. Also thanks to the sandwich PhD students and guest researchers, who even in their short stay added to the good atmosphere of the group. Lunch in the coffee corner or on the roof on a nice day with all of you was great fun, thank you for making the atmosphere at HPP great. But also thanks for the fun social events: hotpots, the gym girls group, karaoke parties, drinking at the Vlaamse Reus (you know who you are ;)), PhD parties, pizza parties, noodles, going out in Nijmegen, visits to Amsterdam. You guys made this PhD an experience I will never forget. Thank you so much and all the best with your PhDs and future scientific careers. Let me not forget the rest of the staff at HPP, Julian (for the gezellige lunches), Wim, Jeremy, Pauline, Rob, Maarten, Pol. Although I didn't directly work with you, you were always there for advice and questions or just a social chat. Thanks so much. To the new colleagues that arrived after I started, good luck, you are in good hands.

Thanks to the Plant Breeding group. Although I was not officially in the group I was allowed to use your lab. Thank you for your help and the patience for helping me around the place and for occasionally giving me cake (Mas!). Alma, thanks for the talks on the train and for introducing me to Flor-id!

Thank you Lennart and Claudius from PE-RC for the help with the whole PhD trajectory and especially for giving me renewed courage to continue with the project after a tough period. Claudie and Fenny, thank you for our talks, they helped.

And last, but absolutely not least a big thanks to my family and friends. Mom, you always had good advice for me whether I wanted it or not :) your TGIFs were the perfect celebration of the weekend after a difficult week, and although you were far away, you felt very close. Bart, thank you for your good advice and relaxed attitude when I was stressing myself out too much. Azinta, thank you for letting me complain a LOT! For sharing work horrors, tough times, small victories and anything else. Sissa-wing, you are my rock, stay tough (haha cheesy) and thank you so much for helping me with the night measurements. Maud, cuz, second sister, thank you for always having my back and even from the

other side of the world for being there for me when I needed it (and when you were in NL for helping me with the impossible harvest). De Ent (MC), second mother, thanks for always having good advice, being my second home, being a great example and just being de Ent. Reigahs (Jane and Rits), thank you for being there and Opper Reigah Rits, thank you for being my inspiration PhD! The next Plantenga PhD party will be yours. To the rest of my family, thanks for making my life a better place by just being there. Ex-snor and Dorien, thanks for allowing me to use your house to write some of this thesis. Schone familie, Corry, Martin, Remon and Chiara. Thanks for listening to my potato stories and not letting it show if you were bored ;) And of course a special thanks to my friends! Sophie, Annemarijn (studying/writing together at the end!), Sabine, Irene, Thomas, Roderick, Annerieke, Katja, Hanneke, Judith, Margit. For the many talks, patience with my changing moods, diversion from the PhD work and just for making me realize and remember that there is (much) more to life than a PhD project. Thanks to my biologist friends, Irene, Paula and Iris, for sharing the passion with me and for showing me how to successfully finish a PhD project.

If I forgot anyone, my apologies. I would like to thank everyone who helped me, inspired me or was just there for me these past 4 and a half years. This PhD would not have been the same without you.

About the author

Faline Dawn-Marie Plantenga was born in Malang, Indonesia on the 11th of May, 1987. Due to her father Jaap Plantenga's job as a tropical agronomist, Faline and her family (mother Marcia and sister Azinta) travelled around the world. After having lived in the Netherlands, India and Jamaica (Marcia's home country), Faline's family moved back to the Netherlands when she was ten.

Faline attended the Stedelijk Gymnasium Arnhem where she chose the profile Nature and Health with the additional subjects Economics and Art. After graduating, Faline decided she would study Industrial design in Delft, but first take a year off to study Italian in Milan with her cousin Maud Vis and enjoy the independent life. Life in the big city made Faline realize she wanted to work with nature and not be stuck in an office her whole life. She therefore started studying Biology at the University of Utrecht. Early on she realized she was very interested in plants and wanted to continue working with plants in the future. This decision was reinforced after a practical where she and her fellow students had to dissect rats (which were still warm). After this, she focused on plant subjects and was very happy with this choice as she became very fascinated by these organisms.

After finishing her bachelor studies in the summer of 2009, Faline wanted to visit her family. Her sister was living in Beijing, China at the time, and her mom and stepfather Bart Koolhoven were living on their farm in Jamaica. As Jamaica and China were not exactly next to each other, Faline decided to buy an around the world ticket and take some time off to visit her family and see a bit of the world. During this trip she also visited her friend Irene Bender in Costa Rica, and here the idea came about to do a master thesis project together once they started their Master Environmental Biology at the University of Utrecht. In 2010 Irene and Faline started their research project (supervisor Hans ter Steege) and after three months of preparations they travelled to the Amazonian rainforest in Colombia (Leticia) to determine the vertical distribution of epiphytes throughout trees. To sample the epiphytes they had to travel to an isolated research station in the Amazon rainforest, 5 hours walk from the nearest village. Here they spent two months, cut off from the outside world in a very basic camp.

To sample the epiphytes, Irene and Faline used a bow and arrow to shoot a climbing rope into the trees after which they could climb up using canyoneering gear and sample the epiphytes on the trunk and in the canopy. After identifying the plants for a month in Bogota, Faline completed her major research project after which she followed courses and wrote her literature thesis on the interactions between arbuscular mycorrhizal fungi and their host plants (supervisor Corné Pieterse). She wrapped up her master studies with a final research project at research station Agroscope in Zurich (supervisor Marcel van der Heijden). Here she studied whether arbuscular mycorrhizal fungi could be used to reduce nutrient losses from soils.

After completing her master studies in 2012, Faline decided she would never do a PhD and started working for a young-plant company (Florensis) as a research assistant. After one and a half year in the R&D department, and having learnt a lot about horticulture, Faline's need for a scientific challenge grew and she decided that a PhD might not be such a bad idea. She soon found a very interesting position at Wageningen University & Research and started a project on the environmental effect on potato flowering and tuberization in May 2014. The results of her PhD research are described in this thesis.

At the moment Faline is living in Amsterdam with her partner Stefan van den Heuvel and has just started her new adventure as a plant physiologist at WUR Glastuinbouw.

PhD Education Certificate

PE&RC Training and Education Statement
With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)
- Unravelling the enigma of the dual reproduction strategy in potato (2018)

Writing of project proposal
- Unravelling the enigma of dual propagation strategy of plants (2014)

Post-graduate courses (5 ECTS)
- Applied methods in crop physiology; Aarhus University, Denmark (2014)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Plant Biology: Light spectral effects on potato plants (2018)

Deficiency, Refresh, Brush-up courses (2.5 ECTS)
- Basic statistics (2014)
- Distance learning course Plant Breeding: principles of plant breeding (2015)

Competence strengthening / skills courses (3.4 ECTS)
- PhD Competence Assessment; WGS (2014)
- PhD workshop carousel; WGS (2015)
- Scientific writing; In'to Languages (2016)
- Career Orientation; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- PE&RC First years weekend (2014)
- PE&RC Day (2014-2016)
- PE&RC Mid-term weekend (2016)

Discussion groups / local seminars / other scientific meetings (8.2 ECTS)

- FLOP: frontier literature in plant physiology (2014-2018)
- NWO-ALW; poster presentation; Lunteren (2015)
- Plant control by LED light; Wageningen (2015, 2017)

International symposia, workshops and conferences (4.4 ECTS)

- ISHS – VIII International Symposium on Light in Horticulture – East-Lansing; oral presentation; Michigan, USA (2016)
- XIV Solanaceae and III Cucurbitaceae Genomics Joint Conference; poster presentation; Valencia, Spain (2017)

Lecturing / Supervision of practicals / tutorials (3.6 ECTS)

- Concepts in environmental plant physiology (2014)
- Physiology and development of horticultural plants (2015-2017)

Supervision of MSc students (3 ECTS)

- Melpomeni Siakou: effect of far-red light on potato

The research described in this thesis was financially supported by the Netherlands Organization for Scientific Research (NWO), with financial contributions from Signify, Solynta, Beekenkamp Plants and Incotec.

Cover design and illustrations
Stefan van den Heuvel
www.stefanvandenheuvel.nl

Printed by
www.dekijm.nl (cover)
www.proefschriftmaken.nl (book)